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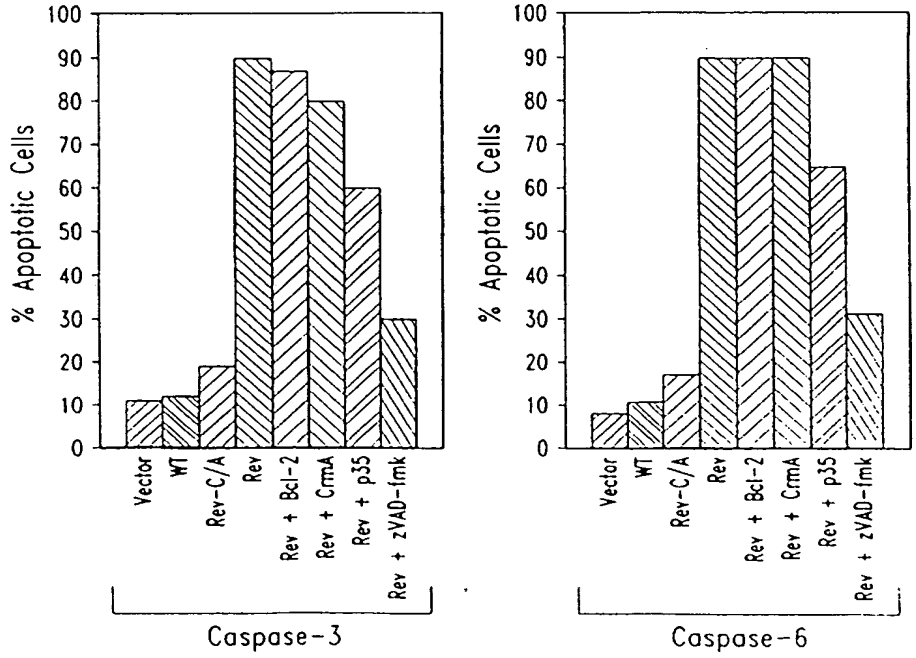

(57) Abstract

Rev caspases comprising a primary product in which the small subunit is N terminal to the large subunit are provided. Rev caspases are used for screening and identifying caspase inhibitors and enhancers. Rev caspase genes can be delivered to cells for gene therapy.

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RECOMBINANT, ACTIVE CASPASES AND USES THEREOF

TECHNICAL FIELD

The present invention relates generally to regulating apoptosis, and more particularly to the novel aspartate-specific cysteine proteases known as caspases, their coding regions, mutant forms thereof, and their use in screening assays and as pharmaceutical compositions for the controlled death of targeted cells to treat human disease.

BACKGROUND OF THE INVENTION

Tissue homeostasis is maintained by the process of apoptosis—that is, the normal physiological process of programmed cell death. Changes to the apoptotic pathway that prevent or delay normal cell turnover can be just as important in the pathogenesis of diseases as are abnormalities in the regulation of the cell cycle. Like cell division, which is controlled through complex interactions between cell cycle regulatory proteins, apoptosis is similarly regulated under normal circumstances by the interaction of gene products that either prevent or induce cell death.

Since apoptosis functions in maintaining tissue homeostasis in a range of physiological processes such as embryonic development, immune cell regulation and normal cellular turnover, the dysfunction or loss of regulated apoptosis can lead to a variety of pathological disease states. For example, the loss of apoptosis can lead to the pathological accumulation of self-reactive lymphocytes that occurs with many autoimmune diseases. Inappropriate loss or inhibition of apoptosis can also lead to the accumulation of virally infected cells and of hyperproliferative cells such as neoplastic or tumor cells. Similarly, the inappropriate activation of apoptosis can also contribute to a variety of pathological disease states including, for example, acquired immunodeficiency syndrome (AIDS), neurodegenerative diseases and ischemic injury. Treatments which are specifically designed to modulate the apoptotic pathways in these and other pathological conditions can alter the natural progression of many of these diseases.

Although apoptosis is mediated by diverse signals and complex interactions of cellular gene products, the results of these interactions ultimately feed into a cell death pathway that is evolutionarily conserved between humans and invertebrates. The pathway, itself, is a cascade of proteolytic events analogous to that of the blood coagulation cascade.

Several gene families and products that modulate the apoptotic process have now been identified. One family is the aspartate-specific cysteine proteases ("caspases"). The caspase Ced-3, identified in *C. elegans*, is required for programmed cell death during development of the roundworm *C. elegans*. Ced-3 homologues as well as other caspases have been characterized. The human caspase family includes, for example, human ICE (interleukin-1-ß converting enzyme) (caspase-1), $ICE_{rel}II$ (caspase-4), $ICE_{rel}III$ (caspase-5), Mch5 (caspase-8), Mch4 (caspase-10), ICE-LAP6 (caspase-9), Mch2 (caspase-6), CPP32 (caspase-3), ICE-LAP3 (casepase-7), ICH-1 (caspase-2), Caspase 11-14, and others.

The caspases share many features. In this regard, caspases are cysteine proteases (named for a cysteine residue in the active site) that cleave substrates at Asp-X bonds. Furthermore, the primary caspase product is a zymogen that requires proteolytic cleavage at specific internal aspartate residues for activation. The primary gene product is arranged such that the N-terminal peptide (prodomain) precedes a large subunit domain, which precedes a small subunit domain. Cleavage of a caspase yields two subunits, a large (generally approximately 20 kD) and a small (generally approximately 10 kD) subunit that associate non-covalently: to form a heterodimer, and, in some caspases, an N-terminal peptide of varying length (see Figure 1). The heterodimer may combine non-covalently to form a tetramer.

Caspase zymogens are themselves substrates for caspases. Inspection of the interdomain linkages in each zymogen reveals target sites (i.e. protease sites) that indicate a hierarchical relationship of caspase activation. By analyzing such pathways, it has been demonstrated that caspases are required for apoptosis to occur. Moreover, caspases appear to be necessary for the accurate and limited proteolytic events which are the hallmark of classic apoptosis (see Salvesen and Dixit, *Cell*

91:443-446, 1997). However, when overexpressed in mammalian cells, the short prodomain caspases-3 and -6 cells are unable to undergo autocatalytic processing/activation and do not induce apoptosis. Thus, no cellular model system has been developed in which to test inhibitors of these caspases nor is gene delivery of a caspase commonplace.

Therefore, there exists a need in the art for methods of assaying compounds for their ability to affect caspase activity as well as for methods of regulating caspases in order to treat diseases and syndromes. The present invention provides recombinant caspase constructs that are active in cells, allowing the regulation of apoptosis for the treatment of pathology as well as providing methods and compositions for assaying compounds for caspase inhibitory and, thus, anti-apoptotic effects, while further providing other related advantages.

SUMMARY OF THE INVENTION

The present invention generally provides rev-caspases. In one aspect, the invention provides an isolated nucleic acid molecule encoding a rev-caspase. In certain embodiments, the rev-caspase is selected from the group consisting of rev-caspase-1, rev-caspase-2, rev-caspase-3, rev-caspase-4, rev-caspase-5, rev-caspase-6, rev-caspase-7, rev-caspase-8, rev-caspase-9, rev-caspase-10, rev-caspase-11, rev-caspase-12, rev-caspase-13, and rev-caspase-14. In other preferred embodiments, the rev-caspase is a human rev-caspase. Nucleic acid and amino acid sequences of rev-caspases are provided. The invention also provides rev-caspase proteins.

In another aspect, an expression vector comprising the nucleic acid molecule encoding rev-caspase is provided, wherein the sequence encoding rev-caspase is operatively linked to a promoter. In certain embodiments, the promoter is inducible, such as HIV LTR. Host cells transfected with the expression vectors are also provided.

In the present invention, methods of identifying an inhibitor or enhancer of caspase processing activity are provided, comprising: (a) contacting a sample containing an *in vitro* translated rev-caspase with a candidate inhibitor or

candidate enhancer; and (b) detecting the presence of large and small subunits of rev-caspase, and therefrom determining the level of caspase processing activity, wherein a decrease in processing indicates the presence of a caspase inhibitor, and wherein an increase in processing indicates the presence of a caspase enhancer, wherein processed rev-caspase yields large and small subunits.

In other aspects, methods are provided for identifying an inhibitor or enhancer of caspase processing activity, comprising: (a) contacting a cell transfected with the vector expressing rev-caspase with a candidate inhibitor or candidate enhancer; and (b) detecting the presence of large and small subunits of rev-caspase, and therefrom determining the level of caspase processing activity, wherein a decrease in processing indicates the presence of a caspase inhibitor, and wherein an increase in processing indicates the presence of a caspase enhancer, wherein processed rev-caspase yields large and small subunits.

Methods are also provided for identifying an inhibitor or enhancer of caspase-mediated apoptosis, comprising: (a) contacting a cell transfected with the vector expressing rev-caspase with a candidate inhibitor or candidate enhancer or with a reference compound; and (b) detecting cell viability, wherein viability of cells contacted with a candidate is increased in the presence of an inhibitor and is decreased in the presence of an enhancer compared to cells contacted with a reference compound.

In other aspects, gene delivery vehicles, comprising the nucleic acid molecule encoding a rev-caspase are provided, wherein the rev-caspase sequence is operatively linked to a promoter. In preferred embodiments, the gene delivery vehicle is a retrovirus or adenovirus or the nucleic acid molecule is associated with a polycation. The gene delivery vehicle may further comprise a ligand that binds a cell surface receptor.

The invention also provides methods of treating cancer or autoimmune diseases, comprising administering to a patient the gene delivery vehicles disclosed herein.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, the various references set forth below that describe in more detail certain procedures or compositions (*e.g.*, plasmids, etc.), and are therefore incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a schematic representation of the processing and folding of human caspase-3 into the mature zymogen represented by bar and ribbon diagrams and is representative of other caspases. α helices are shown as spirals and the β strands are represented by arrows. The N- and the C-termini of the LS are labeled N-LS and C-LS, respectively, and the termini of the SS are similarly labeled N-SS and C-SS.

Figures 1B and C are schematic representations of rev-caspase-3 and -6, respectively. In both Figures 1B and C the N-terminus of the SS and the C-terminus of the LS are labeled as in Figure 1A and the linker region between the C-SS and the N-LS which includes the caspase-3 or -6 prodomain is represented by a thin line. Solid arrows indicate the cleavage sites (DEVDG, Asp9 and Asp28—for rev-caspase-3 and VEIDA and Asp23—for rev-caspase-6) within the linker region. The hatched boxes represent a 15 residue-long T7-tag on the N-termini of the wild-type and the rev-caspases. All aspartate processing sites are indicated on the bar diagrams. Figure 1B further depicts a schematic representation of the spontaneous folding of rev-caspase-3 into the mature zymogen represented a ribbon diagram. The ribbon diagram of rev-caspase-3 is based on the published crystal structure of caspase-3.

Figure 2A is a scanned image of an autoradiogram representing SDS-PAGE analysis of rev-caspase autoprocessing. Caspase-3 and -6 or their rev-versions (Rev) including active site Cys to Ala mutants (Rev C/A) in pRSC-lacZ constructs were *in vitro* translated in the presence of ^{35}S-methionine. The translation products

were then analyzed by SDS-PAGE and autoradiography. The LS and the SS are indicated.

Figures 2B and C are scanned images of autoradiograms representing SDS-PAGE analysis of the autoprocessing of rev-caspase-3 and -6, respectively in the presence of varying levels of selected caspase inhibitors. Rev-caspase-3 (Figure 2B) or rev-caspase-6 (Figure 2C) were *in vitro* translated in the presence of increasing concentrations of DEVD-CHO (0.04 μM) or zVAD-fmk (0-5 μM). The translation products were then analyzed as in Figure 2A. WT, wild-type.

Figures 3A and B are scanned images representing the SDS-PAGE analysis of the ability of rev-caspase-3 (imaged by western blot) and -6 (imaged by autoradiogram) to cleave PARP and lamin, respectively. In Figure 3A purified human PARP was incubated with buffer (lane 1) or BL-21 bacterial extracts prepared from bacteria transformed with caspase-3 (lane 2), rev-caspase-3 (lane 3), caspase-6 (lane 4), rev-caspase-6 (lane 5) constructs or empty pET28a vector (lane 6) for 2 h at 37°C. The reaction products were then analyzed by SDS-PAGE and Western blotting with anti-human PARP antibody. In Figure3B a cDNA encoding the C-terminus of lamin A (amino acids) which contain the caspase-6 cleavage site (VEIDA) was amplified by PCR and *in vitro* translated in the presence of ^{35}S-methionine. The labeled product was incubated with buffer (lane 1) or the BL-21 bacterial extracts listed above for 2 h at 37°C, and then analyzed by SDS-PAGE and autoradiography. The cleavage products are indicated to the right.

Figures 4A and B are bar diagrams representing the ability of rev-caspase-3 and -6 to induce apoptosis in MCF-7 cells. MCF-7 cells were transiently transfected with either rev-caspase-3 (Figure 4A), or rev-caspase-6 (Figure 4B) expression constructs in combination with 4-fold of CrmA, p35 or Bcl-2 expression constructs, or 20 μM zVAD-fmk. Cells transfected with an empty vector or the wild-type caspase-3 or -6 were used as controls.

Figure 5A is a scanned image of an autoradiogram representing SDS-PAGE analysis of the enzymatic activity of uncleavable rev-caspase-3. Uncleavable rev-caspase-3 was *in vitro* translated in the absence or the presence of increasing

concentrations of DEVD-CHO. The translation product contains a cleavable 35 residues-long His6-T7-tag at its N-terminus. The active site mutant rev-caspase-3 (Rev C/A) was used as a control. The p32 cleavage product without the His6-T7-tag is indicated to the right.

Figure 5B is a plot of an activity assay of bacterially expressed uncleavable rev-caspase-3. The plot measures the ability of the uncleavable rev-caspase-3 to cleave the DEVD-AMC substrate. Rev, rev-caspase-3; Rev-mod, uncleavable rev-caspase-3; Rev-C/A, rev-caspase-3 with an active site mutation.

Figure 6 is a multiple amino acid sequence alignment of the relatively conserved regions of the caspases (SEQ ID NOs: 54-115). In the bottom line, "c" refers to residues involved in catalysis, "b" refers to residues that bind the substrate-carboxylate of P1 Asp, "a" refers to residues adjacent to the substrate P2-P4 recognition responsible amino acids, "DX" indicates known and potential processing sites between the small and large subunits of the caspases. The roman numerals at the left of the figure indicate the caspase subfamilies: Ced-like (I), ICE-like (II), and the Nedd2/Ich-1-like (III). The asterisk represents the non-conservative substitution in the active site pentapeptide sequences of Mch4 (caspase-10), Mch5 (caspase-8), and Mch6 (caspase-9)

Figure 7 depicts a nucleotide sequence of Rev-caspase-3 (SEQ ID NO:1).

Figure 8 depicts a nucleotide sequence of uncleavable Rev-caspase-3 (SEQ ID NO:2).

Figure 9 depicts a nucleotide sequence of Rev-caspase-6 (SEQ ID NO:3).

Figure 10 depicts a schematic of some possible rev-caspases. I, intervening sequence; SS, small subunit; P, prodomain; LS, large subunit; X, linker.

Figures 11A and 11B depict a nucleotide (SEQ ID NOs: 4 and 5) and predicted amino acid sequence of caspase-1 (SEQ ID NO:6).

Figures 12A and 12 B depict a nucleotide (SEQ ID NOs: 7 and 8) and predicted amino acid sequence of caspase-2 (SEQ ID NO:9).

Figures 13A and 13B depict a nucleotide (SEQ ID NOs: 10 and 11) and predicted amino acid sequence of caspase-3 (SEQ ID NO:12).

Figures 14A and 14B depict a nucleotide (SEQ ID NOs: 13 and 14) and predicted amino acid sequence of caspase-4 (SEQ ID NO:15).

Figures 15A and 15B depict a nucleotide (SEQ ID NOs: 16 and 17) and predicted amino acid sequence of caspase-5 (SEQ ID NO:18).

Figures 16A and 16B depict a nucleotide (SEQ ID NOs: 19 and 20) and predicted amino acid sequence of caspase-6 (SEQ ID NO:21).

Figures 17A and 17B depict a nucleotide (SEQ ID NOs: 22 and 23) and predicted amino acid sequence of caspase-7 (SEQ ID NO:24).

Figures 18A-18C depict a nucleotide (SEQ ID NOs: 25 and 26) and predicted amino acid sequence of caspase-8 (SEQ ID NO:27).

Figures 19A and 19B depict a nucleotide (SEQ ID NOs: 28 and 29) and predicted amino acid sequence of caspase-9 (SEQ ID NO: 30).

Figures 20A and 20B depict a nucleotide (SEQ ID NOs: 31 and 32) and predicted amino acid sequence of caspase-10 (SEQ ID NO:33).

Figures 21A, 21B, and 21C depict predicted amino acid sequences of Rev-caspase-3 (A; SEQ ID NO:34), uncleavable rev-caspase-3 (B; SEQ ID NO:35), and rev-caspase-6 (C; SEQ ID NO:36).

DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms that will be used hereinafter.

As used herein, a caspase refers to a cysteine protease that specifically cleaves proteins after Asp residues. Caspases are initially expressed as zymogens, in which a large subunit is N-terminal to a small subunit. Caspases are generally activated by cleavage at internal Asp residues (Figure 1A). These proteins have been identified in many eukaryotes, including *C. elegans*, *Drosophila*, mouse, and humans. Currently, there are at least 14 known caspase genes, named caspase-1 through caspase-14. Caspases are found in myriad organisms, including human, mouse,

insect (*e.g., Drosophila*), and other invertebrates (*e.g., C. elegans*). In Table 1, ten human caspases are listed along with their alternative names. The nucleotide and amino acid sequences of representative human caspase gene products are presented in SEQ ID NOs: 4-33 and Figures 11-20.

Caspase	Alternative name
Caspase-1	ICE
Caspase-2	ICH-1
Caspase-3	CPP32, Yama, apopain
Caspase-4	$ICE_{rel}II$; TX, ICH-2
Caspase-5	$ICE_{rel}III$; TY
Caspase-6	Mch2
Caspase-7	Mch3, ICE-LAP3, CMH-1
Caspase-8	FLICE; MACH; Mch5
Caspase-9	ICE-LAP6; Mch6
Caspase-10	Mch4, FLICE-2

As used herein, "rev-caspase" refers to a cysteine protease that specifically cleaves proteins after Asp residues and is expressed as a zymogen, in which a small subunit is N-terminal to a large subunit.

Within the context of this invention, it should be understood that a caspase or rev-caspase includes wild-type protein sequences, as well as other variants (including alleles) of the native protein sequence. Briefly, such variants may result from natural polymorphisms or may be synthesized by recombinant methodology, and differ from wild-type protein by one or more amino acid substitutions, insertions, deletions, or the like. Typically, when engineered, amino acid substitutions will be conservative, *i.e.*, substitution of amino acids within groups of polar, non-polar, aromatic, charged, etc. amino acids. In the region of homology to the native sequence, variants should preferably have at least 90% amino acid sequence identity, and within certain embodiments, greater than 92%, 95%, or 97% identity.

As will be appreciated by those skilled in the art, a nucleotide sequence encoding a caspase, rev-caspase or variant may differ from the known native sequences, due to codon degeneracies, nucleotide polymorphisms, or amino

acid differences. In other embodiments, variants should preferably hybridize to the native nucleotide sequence at conditions of normal stringency, which is approximately 25-30°C below Tm of the native duplex (*e.g.*, 5X SSPE, 0.5% SDS, 5X Denhardt's solution, 50% formamide, at 42°C or equivalent conditions; *see generally*, Sambrook et al. *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Press, 1987; Ausubel et al., *Current Protocols in Molecular Biology*, Greene Publishing, 1987). Low stringency hybridizations utilize conditions approximately 40°C below Tm, and high stringency hybridizations utilize conditions approximately 10°C below Tm. Variants preferably have at least 75% nucleotide identity to native sequence, preferably at least 80%, 85%, and most preferably at least 90% nucleotide identity.

An "isolated nucleic acid molecule" refers to a polynucleotide molecule in the form of a separate fragment or as a component of a larger nucleic acid construct, that has been separated from its source cell (including the chromosome it normally resides in) at least once in a substantially pure form. Nucleic acid molecules may be comprised of a wide variety of nucleotides, including DNA, RNA, nucleotide analogues, or some combination of these.

A. CASPASE AND REV-CASPASE GENES AND GENE PRODUCTS

As noted above, the invention provides compositions relating to caspase and rev-caspase genes and gene products, and methods for the use of the genes and gene products. In particular, the invention provides rev-caspase constructs that are active when expressed in cells. Given the disclosure provided herein, a caspase gene can be isolated from a variety of cell types and engineered to produce a rev-caspase.

1. Isolation of caspase genes

The present invention, as described herein, provides rev-caspase genes, which are constructed from caspase genes. Caspase genes may be isolated from either genomic DNA or preferably cDNA. Isolation of caspase genes from genomic DNA or cDNA typically can proceed by, first, generating an appropriate DNA library

through techniques for constructing libraries that are known in the art (*see* Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, 1989) or purchased from commercial sources (*e.g.*, Clontech, Palo Alto, CA). Briefly, cDNA libraries can be constructed in bacteriophage vectors (*e.g.*, λZAPII), plasmids, or others, which are suitable for screening, while genomic DNA libraries can be constructed in chromosomal vectors, such as YACs (yeast artificial chromosomes), bacteriophage vectors, such as λEMBL3, λgt10, cosmids, or plasmids.

In one embodiment known caspase sequences may be utilized to design an oligonucleotide hybridization probe suitable for screening genomic or cDNA libraries. Preferably, such oligonucleotide probes are 20-30 bases in length. To facilitate hybridization detection, the oligonucleotide may be conveniently labeled, generally at the 5' end, with a reporter molecule, such as a radionuclide, (*e.g.*, ^{32}P), enzymatic label, protein label, fluorescent label, or biotin. Such libraries are then generally plated as phage or colonies, depending upon the vector used. Subsequently, a nitrocellulose or nylon membrane, to which the colonies or phage have been transferred, is probed to identify candidate clones which contain the caspase gene. Such candidates may be verified as containing caspase DNA by any of various means including, for example, DNA sequence analysis or hybridization with a second, non-overlapping probe.

Once a library is identified as containing a caspase gene, the gene can be isolated by amplification. Briefly, when using cDNA library DNA as a template amplification primers are designed based upon known caspase gene sequences (see GenBank Accession Nos. X65019 (caspase-1), U13021 (caspase-2), U13737 (caspase-3), U25804 (caspase-4), U28015 (caspase-5), U20536 (caspase-6), U37448 (caspase-7), U60520 (caspase-8), U56390 (caspase-9), U60519 (caspase-10) Y13089 (caspase-11), Y13090 (caspase-12), AF078533 (caspase-13), AF092997 (caspase-14), and sequences provided herein). Amplification of cDNA libraries made from cells with high caspase activity is preferred.. Primers for amplification are preferably derived from sequences in the 5' and 3' untranslated region in order to isolate a full-length cDNA. The primers preferably have a GC content of about 50% and contain

restriction sites to facilitate cloning and do not have self-complementary sequences nor do they contain complementary sequences at their 3' end (to prevent primer-dimer formation). The primers are annealed to cDNA or genomic DNA and sufficient amplification cycles are performed to yield a product readily visualized by gel electrophoresis and staining. The amplified fragment is purified and inserted into a vector, such as λgt10 or pBS(M13+), and propagated. Confirmation of the nature of the fragment is obtained by DNA sequence analysis or indirectly through amino acid sequencing of the encoded protein.

Other methods may also be used to obtain a caspase encoding nucleic acid molecule. For example, a nucleic acid molecule encoding caspase may be obtained from an expression library by screening with an antibody or antibodies reactive to caspase (*see*, Sambrook, et al. *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, NY, 1987; Ausubel, et al. *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley-Interscience, NY, 1995).

Caspase genes from a variety of species may be isolated using the compositions provided herein. For closely related species, the human sequence or portion thereof may be utilized as a probe on a genomic or cDNA library. For example, a fragment of caspase that encompasses the catalytic site may be labeled and used as a probe on a library constructed from mouse, primate, rat, dog, or other vertebrate, warm-blooded or mammalian species. An initial hybridization at normal stringency may yield candidate clones or fragments. If no hybridization is initially observed, varying degrees of stringency may be used. (see Sambrook et al. *supra*, and other well-known sources for stringency conditions) While such probes may also be used to probe libraries from evolutionarily diverse species, such as *Drosophila*, hybridization conditions will likely be more relaxed.

While relaxed hybridization conditions using probes designed from human sequences may identify caspase genes of evolutionarily diverse species it may be more beneficial to attempt to directly isolate these genes from a library using methods which do not require the human sequence per se. These methods include,

but are not limited to, amplification using primers derived from conserved areas, amplification using degenerate primers from various regions, antibody probing of expression libraries, and the like. For example, random-primed amplification (*e.g.*, polymerase chain reaction) may be employed (*see, e.g., Methods Enzymol. 254*: 275, 1995; *Trends Genet. 11*: 242, 1995; Liang and Pardee, *Science 257*: 967, 1992; Welsh et al., *Nucl. Acids Res. 20*: 4965, 1992). In addition, variations of random-primed PCR may also be used, especially when a particular gene or gene family is desired. In such a method, one of the amplification primers is an "anchored oligo(dT) (oligo(dT)dN)" and the other primer is a degenerate primer based upon amino acid or nucleotide sequence of a related gene. A gene sequence is identified as a caspase by amino acid similarity and / or nucleic acid similarity. Generally, amino acid similarity is preferred. Candidate caspase genes are examined for enzyme activity by one of the functional assays described herein or other equivalent assays.

Variants of caspase and rev-caspase genes provided herein may be engineered from natural variants (*e.g.*, polymorphisms, splice variants, mutants), synthesized or constructed. Many methods have been developed for generating mutants (*see, generally*, Sambrook et al., *supra*; Ausubel, et al., *supra*, and the discussion above). Briefly, preferred methods for generating a few nucleotide substitutions utilize an oligonucleotide that spans the base or bases to be mutated and contains the mutated base or bases. The oligonucleotide is hybridized to complementary single stranded nucleic acid and second strand synthesis is primed from the oligonucleotide. The double-stranded nucleic acid is prepared for transformation into host cells, typically *E. coli*, but alternatively, other prokaryotes, yeast or other eukaryotes. Standard screening and vector growth protocols are used to identify mutant sequences and obtain high yields.

Similarly, deletions and/or insertions of the caspase or rev-caspase genes may be constructed by any of a variety of known methods as discussed *supra*. For example, the gene can be digested with restriction enzymes and religated such that a sequence is deleted or religated with additional sequences such that an insertion or large substitution is made. Other means of generating variant sequences may be

employed with methods known in the art, for example those described in Sambrook et al. (*supra*) and Ausubel et al. (*supra*). Verification of variant sequences is typically accomplished by restriction enzyme mapping, sequence analysis, or probe hybridization. Variants which catalyze Asp-specific cleavages are useful in the context of this invention.

B. REV-CASPASES

The caspases of the present invention are generated by rearranging the gene sequence of the caspase gene such that the nucleic acid sequence encoding the small subunit precedes (is 5' to) the nucleic acid sequence encoding the large subunit. These rearranged caspases are called rev-caspases.

1. Structure of rev-caspases

The rev-caspases of the present invention comprise at least a portion of the small subunit and at least a portion of the large subunit. In preferred embodiments, the prodomain or a portion thereof (see Figures 1, 10) and/or an intervening sequence or a portion thereof (see Figure 10) are also present in rev-caspase. In other preferred embodiments, a "linker" region is located between the small and large subunits.

The boundaries of the small subunit and large subunit are identified either experimentally by amino acid sequence analysis of the mature caspase or by inspection of structural homology (*e.g.*, the conserved Asp-X cleavage site). For exemplary purposes, the Table below presents the boundaries of the prodomain (P), large subunit (LS), intervening sequence (I), and small subunit (SS) of human caspase-1 through -10. The nucleotide numbers refer to the nucleotides in SEQ ID NOs: 4-33 and in Figures 11-20.

Caspase	Prodomain	Large Subunit	Intervening sequence	Small Subunit
Caspase-1	1-357	358-891	892-948	949-1212
Caspase-2	1-456	457-948	949-990	991-1305
Caspase-3	1-84	85-525		526-831

Caspase-4	1-240	241-810	811-867	868-1131
Caspase-5	1-363	364-933		934-1254
Caspase-6	1-69	70-537	538-579	580-879
Caspase-7	1-69	70-594		595-909
Caspase-8	1-681	682-1173		1174-1488
Caspase-9	1-390	391-945	946-990	991-1248
Caspase-10	1-657	658-1116		1117-1437

As noted above, a portion of the large subunit and small subunit may be used in rev-caspase constructs. When designing rev-caspases that contain a portion of these subunits, the active site (*e.g.*, QACXG, where X is Arg, Gln, or Gly), which is located near the C-terminus of the large subunit should not be deleted if protease activity is desired. Preferably, the 3-dimensional structure as determined by X-ray crystallography (see Mittl et al., *J. Biol. Chem.*, 272:6539-6547, 1997; Rotondu et al., *Nat. Struct. Biol.*, 3:619-625, 1996; Walker et al., *Cell*, 78:343-352, 1994; Wilson et al., *Nature*, 370:270-275, 1994) is maintained. For example, from the x-ray crystallographic structures of caspases, the amino acids, that are important in binding substrates have been identified. Likewise, substitutions of amino acids in the active site may be detrimental to maintaining activity. Although it is preferred that both subunits are derived from the same caspase, combinations of subunits from different caspases and/or from different species may be used.

The prodomain (sometimes called an N-terminal peptide) is generally not required for enzyme activity and is normally released *in vivo*. Rev-caspases of the present invention optionally have a prodomain or portion thereof. Similarly, the intervening sequence, which is present in certain caspases, is optional for inclusion in rev-caspases.

In certain embodiments, a linker region is engineered between the small and large subunits. A "linker region", as used herein, refers to a peptide of from about 5 to about 50 amino acids. In preferred embodiments, the linker may contain a protease sensitive or cleavage site. Any site recognized by an intracellular protease may be used. In addition, multiple protease sensitive sites may be tandemly

arranged in the same linker. Preferred protease sensitive sites are susceptible to cleavage by caspases or by viral proteases. Preferred caspase sensitive sites include, but are not limited to DXXDG (wherein X is any amino acid; SEQ ID NO:37); DEVDG (SEQ ID NO:38), IETDG (SEQ ID NO:39), YVADG (SEQ ID NO:40), YVHDG (SEQ ID NO:41), and WEHDG (SEQ ID NO:42). Furthermore, the Gly residue may be Ala or another small amino acid. The latter three sites are specifically cleaved by caspases-1, -4, and -5. Other sites specifically cleaved by only one or a few caspases are preferred in certain embodiments. Viral proteases cleavage sites include, but are not limited to, those recognized and cleaved by HIV protease, HCV (hepatitis C virus) protease, HBV (hepatitis B virus) protease, and rhinovirus protease.

2. *Construction of rev-caspases*

Rev-caspases may be constructed from caspase sequences by a variety of methods known in the art. A preferred method is amplification (*e.g.*, polymerase chain reaction (PCR)) to selectively amplify the individual subunits and place these in cloning vectors such as pUC such as described in Example 1. Moreover, such PCR reactions can be performed in a variety of ways such that the primers used for amplification contain specific restriction endonuclease sites to facilitate insertion into a vector.

Further, a variety of other methodologies besides PCR may be used to attain the desired rearrangement. For example, one skilled in the art may employ isothermal methods to amplify the nucleotide sequence of interest, using existing restriction endonuclease sites present in the nucleotide sequence to excise and insert sequences, or by the introduction of distinct restriction endonuclease sites by site-directed mutagenesis followed by excision and insertion. These and other methods are described in Sambrook et al., *supra*; Ausubel, et al., *supra*. Briefly, one methodology is to generate single-stranded cDNA of the caspase of interest, followed by annealing a primer, which is complementary except for the desired alteration (*e.g.*, a small insertion, deletion, or mutation such that a unique restriction site is created between the large and small subunits and/or at the 5' and 3' ends of both subunits).

Bacterial cells are transformed and screened for those which contain desired construct. This construct is then digested to liberate the subunit sequences, which can then be purified and religated into the appropriate orientation.

As indicated above, rev-caspase genes may be manipulated to contain insertions, deletions or substitutions. Moreover, such variant rev-caspase genes useful in the context of this invention include those which facilitate Asp-specific cleavages indicative of caspase activity. Further, variants which are incapable of being cleaved into separate subunits are encompassed within the context of this invention, if those variants are able to facilitate Asp-specific cleavages by way of a cysteine-containing active site. By way of guidance, amino acids involved in catalysis, Asp recognition in substrate, and P2-P4 substrate recognition are provided in Figure 6.

C. VECTORS, HOST CELLS AND MEANS OF EXPRESSING AND PRODUCING PROTEIN

Caspase may be expressed in a variety of host organisms. In certain embodiments, caspase is produced in bacteria, such as *E. coli*, or mammalian cells (*e.g.*, CHO and COS-7), for which many expression vectors have been developed and are available. Other suitable host organisms include other bacterial species, and eukaryotes, such as yeast (*e.g.*, *Saccharomyces cerevisiae*), and insect cells (*e.g.*, Sf9).

A DNA sequence encoding rev-caspase is introduced into an expression vector appropriate for the host. In certain embodiments, rev-caspase is inserted into a vector such that a fusion protein is produced. The rev-caspase sequence is derived as described herein. As discussed above, the sequence may contain alternative codons for each amino acid with multiple codons. The alternative codons can be chosen as "optimal" for the host species. Restriction sites are typically incorporated into the primer sequences and are chosen with regard to the cloning site of the vector. If necessary, translational initiation and termination codons can be engineered into the primer sequences.

At minimum, the vector must contain a promoter sequence. As used herein, a "promoter" refers to a nucleotide sequence that contains elements that direct the transcription of a linked gene. At minimum, a promoter contains an RNA polymerase binding site. More typically, in eukaryotes, promoter sequences contain binding sites for other transcriptional factors that control the rate and timing of gene expression. Such sites include TATA box, CAAT box, POU box, AP1 binding site, and the like. Promoter regions may also contain enhancer elements. When a promoter is linked to a gene so as to enable transcription of the gene, it is "operatively linked".

Other regulatory sequences may be included. Such sequences include a transcription termination signal sequence, secretion signal sequence, origin of replication, selectable marker, and the like. The regulatory sequences are operationally associated with one another to allow transcription or translation.

The expression vectors used herein include a promoter designed for expression of the proteins in a host cell (*e.g.*, bacterial). Suitable promoters are widely available and are well known in the art. Inducible or constitutive promoters are preferred. Such promoters for expression in bacteria include promoters from the T7 phage and other phages, such as T3, T5, and SP6, and the *trp*, *lpp*, and *lac* operons. Hybrid promoters (*see*, U.S. Patent No. 4,551,433), such as *tac* and *trc*, may also be used. Promoters for expression in eukaryotic cells include the P10 or polyhedron gene promoter of baculovirus/insect cell expression systems (*see*, *e.g.*, U.S. Patent Nos. 5,243,041, 5,242,687, 5,266,317, 4,745,051, and 5,169,784), MMTV LTR, CMV IE promoter, RSV LTR, SV40, metallothionein promoter (*see*, *e.g.*, U.S. Patent No. 4,870,009) and the like.

The promoter controlling transcription of rev-caspase may itself be controlled by a repressor. In some systems, the promoter can be derepressed by altering the physiological conditions of the cell, for example, by the addition of a molecule that competitively binds the repressor, or by altering the temperature of the growth media. Preferred repressor proteins include, but are not limited to the *E. coli*

lacI repressor responsive to IPTG induction, the temperature sensitive λcI857 repressor, and the like. The *E. coli* lacI repressor is preferred.

In other preferred embodiments, the vector also includes a transcription terminator sequence. A "transcription terminator region" has either a sequence that provides a signal that terminates transcription by the polymerase that recognizes the selected promoter and/or a signal sequence for polyadenylation.

Preferably, the vector is capable of replication in the host cells. Thus, when the host cell is a bacterium, the vector preferably contains a bacterial origin of replication. Preferred bacterial origins of replication include the f1-ori and col E1 origins of replication, especially the ori derived from pUC plasmids. In yeast, ARS or CEN sequences can be used to assure replication. A well-used system in mammalian cells is SV40 ori.

The plasmids also preferably include at least one selectable marker that is functional in the host. A selectable marker gene includes any gene that confers a phenotype on the host that allows transformed cells to be identified and selectively grown. Suitable selectable marker genes for bacterial hosts include the ampicillin resistance gene (Amp^r), tetracycline resistance gene (Tc^r) and the kanamycin resistance gene (Kan^r). The kanamycin resistance gene is presently preferred. Suitable markers for eukaryotes usually require a complementary deficiency in the host (*e.g.*, thymidine kinase (tk) in tk- hosts). However, drug markers are also available (*e.g.*, G418 resistance and hygromycin resistance).

The sequence of nucleotides encoding rev-caspase may also include a secretion signal, whereby the resulting peptide is a precursor protein processed and secreted. The resulting processed protein may be recovered from the periplasmic space or the fermentation medium. Secretion signals suitable for use are widely available and are well known in the art (von Heijne, *J. Mol. Biol. 184*:99-105, 1985). Prokaryotic and eukaryotic secretion signals that are functional in *E. coli* (or other host) may be employed. The presently preferred secretion signals include, but are not limited to, those encoded by the following *E. coli* genes: pelB (Lei et al., *J.*

Bacteriol. 169:4379, 1987), phoA, ompA, ompT, ompF, ompC, beta-lactamase, and alkaline phosphatase.

One skilled in the art appreciates that there are a wide variety of suitable vectors for expression in bacterial cells and which are readily obtainable. Vectors such as the pET series (Novagen, Madison, WI), the tac and trc series (Pharmacia, Uppsala, Sweden), pTTQ18 (Amersham International plc, England), pACYC 177, pGEX series, and the like are suitable for expression of a rev-caspase. Baculovirus vectors, such as pBlueBac (*see, e.g.*, U.S. Patent Nos. 5,278,050, 5,244,805, 5,243,041, 5,242,687, 5,266,317, 4,745,051, and 5,169,784; available from Invitrogen, San Diego) may be used for expression in insect cells, such as *Spodoptera frugiperda* sf9 cells (*see*, U.S. Patent No. 4,745,051). The choice of a bacterial host for the expression of a rev-caspase is dictated in part by the vector. Commercially available vectors are paired with suitable hosts.

A wide variety of suitable vectors for expression in eukaryotic cells are available. Such vectors include pCMVLacI, pXT1 (Stratagene Cloning Systems, La Jolla, CA); pCDNA series, pREP series, pEBVHis (Invitrogen, Carlsbad, CA). In certain embodiments, rev-caspase gene is cloned into a gene targeting vector, such as pMC1neo, a pOG series vector (Stratagene Cloning Systems).

Rev-caspase is isolated by standard methods, such as affinity chromatography, size exclusion chromatography, metal ion chromatography, ionic exchange chromatography, HPLC, and other known protein isolation methods. (*see generally* Ausubel et al. *supra*; Sambrook et al. *supra*). An isolated purified protein gives a single band on SDS-PAGE when stained with Coomassie blue.

Rev-caspase may be expressed as a hexa-his fusion protein and isolated by metal-containing chromatography, such as nickel-coupled beads. Briefly, a sequence encoding His_6 is linked to a DNA sequence encoding a rev-caspase. Although the His_6 sequence can be positioned anywhere in the molecule, preferably it is linked at the 3' end immediately preceding the termination codon. The fusion may be constructed by any of a variety of methods. A convenient method is amplification of the rev-caspase gene using a downstream primer that contains the codons for His_6.

Purified rev-caspase protein may be used in assays to screen for inhibitory drugs. These assays may be performed *in vitro* or *in vivo* and utilize any of the methods described herein or that are known in the art. The protein may also be crystallized and subjected to X-ray analysis to determine its 3-dimensional structure or used to raise antibodies.

D. USES OF REV-CASPASE GENE AND GENE PRODUCT

1. Inhibitors and enhancers of caspase activity

Candidate inhibitors and enhancers may be isolated or procured from a variety of sources, such as bacteria, fungi, plants, parasites, libraries of chemicals, peptides or peptide derivatives and the like. Inhibitors and enhancers may be also be rationally designed, based on the protein structure determined from X-ray crystallography (see, Mittl et al., *J. Biol. Chem.*, *272*:6539-6547, 1997). In certain preferred embodiments, the inhibitor targets a specific caspase (*e.g.*, caspase-3 and not any other caspases).

Without being held to a particular mechanism, the inhibitor may act by preventing processing of caspase or by preventing enzymatic activity, or by other mechanism. The inhibitor may act directly or indirectly. In preferred embodiments, inhibitors interfere in the processing of the caspase protein. In other preferred embodiments, the inhibitors are small molecules. In a most preferred embodiment, the inhibitors prevent apoptosis. Inhibitors should have a minimum of side effects and are preferably non-toxic. Inhibitors that can penetrate cells are preferred.

In addition, enhancers of caspase activity or expression are desirable in certain circumstances. At times, increasing apoptosis will have a therapeutic effect. For example, tumors or cells that mediate autoimmune diseases are appropriate cells for destruction. Enhancers may increase the rate or efficiency of caspase processing, increase transcription or translation, or act through other mechanisms. As is apparent to one skilled in the art, many of the guidelines presented above apply to the design of enhancers as well.

Screening assays for inhibitors and enhancers will vary according to the type of inhibitor or enhancer and the nature of the activity that is being affected. Assays may be performed *in vitro* or *in vivo*. In general, *in vitro* assays are designed to evaluate caspase protein processing or caspase enzymatic activity, and *in vivo* assays are designed to evaluate caspase protein processing, caspase enzymatic activity, apoptosis, or caspase cleavage of substrate. In any of the assays, a statistically significant increase or decrease compared to a proper control is indicative of enhancement or inhibition.

One *in vitro* assay can be performed by examining the effect of a candidate compound on processing of rev-caspase into two subunits. Briefly, a cleavable form of rev-caspase, that is a primary translation product, is obtained from an *in vitro* translation system. The cleavable form of rev-caspase is preferably constructed to be auto-cleaved, but can be constructed to be cleaved by other protease components present or added to the reaction. This primary product is contacted with or without or translated in the presence or absence of a candidate compound and assessed for appearance of the two subunits. to facilitate detection, typically, the primary product of rev-caspase is labeled during translation, cell viability, and the like. The two subunits may be readily detected by autoradiography after gel electrophoresis. One skilled in the art will recognize that other methods of labeling and detection may be used alternatively.

An alternative *in vitro* assay is designed to measure cleavage of a caspase substrate (*e.g.*, Acetyl DEVD-aminomethyl coumarin (amc), lamin, PRPP, and the like). Substrate turnover may be assayed using either cleavable or noncleavable rev-caspase. Briefly, in this method, rev-caspase is translated and allowed sufficient time to be processed, if a cleavable rev-caspase is being used. The caspase substrate along with the candidate compound is added to the reaction. Detection of cleaved substrate is performed by any one of a variety of standard methods. Generally, the substrate will be labeled and followed by an appropriate detection means.

Moreover, any known enzymatic analysis can be used to follow the inhibitory or enhancing ability of a candidate compound with regard to a rev-caspase of this invention. For example, one could express the rev-caspase of interest in a cell line be it bacterial, insect, mammalian or other, either in cleavable or noncleavable form and purify the rev-caspase. The purified rev-caspase could then be used in a variety of assays to follow its catalytic ability in the presence of candidate compounds, as noted above. Such methods of expressing and purifying recombinant proteins are known in the art and examples can be found in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, 1989 as well as in a number of other sources.

In vivo assays are typically performed in cells transfected either transiently or stably with an expression vector containing a rev-caspase gene, such as those described herein. These cells are used to measure rev-caspase processing, substrate turnover, or apoptosis in the presence or absence of a candidate compound. When assaying apoptosis, a variety of cell analyses may be used including, for example, dye staining and microscopy to examine nucleic acid fragmentation and porosity of the cells. Further, *in vivo* assaying for the ability of the transfected rev-caspase to cleave known substrates that are co-transfected or placed in the cell culture media in the presence of the candidate compound can be performed thereby allowing for the detection and determination of substrate turnover.

The assays briefly described herein may be used to identify an enhance or inhibitor that is specific for an individual caspase. In a preferred embodiment candidate compounds would be analyzed using a variety of rev-caspases (*e.g.*, rev-caspase-1 through rev-caspase-14) to identify specific for individual caspases.

A variety of methodologies exist can be used to investigate the effect of a candidate compound. Such methodologies are those commonly used to analyze enzymatic reactions and include, for example, SDS-PAGE, spectroscopy, HPLC analysis, autoradiography, chemiluminescence, chromogenic reactions, and immunochemistry (*e.g.*, blotting, precipitating, etc.).

Inhibitors and enhancers may be used in the context of this invention to exert control over the cell death process or cytokine activation (e.g., IL-1, which is activated by caspase-1). Thus, these inhibitors and enhancers will have utility in diseases characterized by either excessive or insufficient levels of apoptosis. Inhibitors of proteases have potential to treat the major neurodegenerative diseases: stroke, Parkinson's Disease, Alzheimer's Disease, and ALS. As well, caspase protease inhibitors may be used to inhibit apoptosis in the heart following myocardial infarction, in the kidney following acute ischemia, and in diseases of the liver. In other embodiments, inhibitors of caspase-1 can be used to inhibit the release of the pro-inflammatory IL-1β, and thus may provide therapeutic benefit in treating inflammation and/or autoimmune disorders. Enhancers of caspase activity may be used in contexts when apoptosis or cytokine activation are desired. For example, inducing or increasing apoptosis in cancer cells or aberrantly proliferating cells may be effected by delivery of a caspase enhancer.

The inhibitors and enhancers may be further coupled with a targeting moiety that binds a cell surface receptor specific to the cells. Administration of inhibitors or enhancers will generally follow established protocols. The compounds of the present invention may be administered either alone, or as a pharmaceutical composition. Briefly, pharmaceutical compositions of the present invention may comprise one or more of the inhibitors or enhancers as described herein, in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients. Such compositions may comprise buffers such as neutral buffered saline, phosphate buffered saline and the like, carbohydrates such as glucose, mannose, sucrose or dextrans, mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, chelating agents such as EDTA or glutathione, adjuvants (e.g., aluminum hydroxide) and preservatives. In addition, pharmaceutical compositions of the present invention may also contain one or more additional active ingredients.

Compositions of the present invention may be formulated for the manner of administration indicated, including for example, for oral, nasal, venous,

intracranial, intraperitoneal, subcutaneous, or intramuscular administration. Within other embodiments of the invention, the compositions described herein may be administered as part of a sustained release implant. Within yet other embodiments, compositions of the present invention may be formulized as a lyophilizate, utilizing appropriate excipients which provide stability as a lyophilizate, and subsequent to rehydration.

2. *Gene therapy*

As noted above, rev-caspases may be delivered to cells as part of gene delivery vehicles. In many diseases and syndromes, too little apoptosis is an important feature in their development. Treatment of many autoimmune diseases and tumors would benefit from increased apoptosis. One means to increase apoptosis is to provide target cells with caspase genes in an expressible form. This may be accomplished by delivery of DNA or cDNA capable of *in vivo* transcription of the rev-caspase. More specifically, in order to produce rev-caspases *in vivo*, a nucleic acid sequence coding for the rev-caspase is placed under the control of a eukaryotic promoter (e.g., a pol III promoter, CMV or SV40 promoter). Where it is desired to more specifically control transcription, the rev-caspase may be placed under the control of a tissue or cell specific promoter (*e.g.*, to target cells in the liver), or an inducible promoter, such as metallothionein.

Many techniques for introduction of nucleic acids into cells are known. Such methods include retroviral vectors and subsequent retrovirus infection, adenoviral or adeno-associated viral vectors and subsequent infection, and complexes of nucleic acid with a condensing agent (*e.g.*, poly-lysine). These complexes or viral vectors may be targeted to particular cell types by way of a ligand incorporated into the vehicle. Many ligands specific for tumor cells and other cells are well known in the art.

A wide variety of vectors may be utilized within the context of the present invention, including for example, plasmids, viruses, retrotransposons and cosmids. Representative examples include adenoviral vectors (*e.g.*, WO 94/26914, WO 93/9191; Yei et al., *Gene Therapy* 1:192-200, 1994; Kolls et al., *PNAS*

91(1):215-219, 1994; Kass-Eisler et al., *PNAS 90*(24):11498-502, 1993; Guzman et al., *Circulation 88*(6):2838-48, 1993; Guzman et al., *Cir. Res. 73*(6):1202-1207, 1993; Zabner et al., *Cell 75*(2):207-216, 1993; Li et al., *Hum Gene Ther. 4*(4):403-409, 1993; Caillaud et al., *Eur. J. Neurosci. 5*(10):1287-1291, 1993), adeno-associated type 1 ("AAV-1") or adeno-associated type 2 ("AAV-2") vectors (*see* WO 95/13365; Flotte et al., *PNAS 90*(22):10613-10617, 1993), hepatitis delta vectors, live, attenuated delta viruses and herpes viral vectors (*e.g.*, U.S. Patent No. 5,288,641), as well as vectors which are disclosed within U.S. Patent No. 5,166,320. Other representative vectors include retroviral vectors (*e.g.*, EP 0 415 731; WO 90/07936; WO 91/02805; WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent No. 5,219,740; WO 93/11230; WO 93/10218.

Within certain aspects of the invention, nucleic acid molecules that encode the rev-caspase may be introduced into a host cell utilizing a vehicle, or by various physical methods. Representative examples of such methods include transformation using calcium phosphate precipitation (Dubensky et al., *PNAS 81*:7529-7533, 1984), direct microinjection of such nucleic acid molecules into intact target cells (Acsadi et al., *Nature 352*:815-818, 1991), and electroporation whereby cells suspended in a conducting solution are subjected to an intense electric field in order to transiently polarize the membrane, allowing entry of the nucleic acid molecules. Other procedures include the use of nucleic acid molecules linked to an inactive adenovirus (Cotton et al., *PNAS 89*:6094, 1990), lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA 84*:7413-7417, 1989), microprojectile bombardment (Williams et al., *PNAS 88*:2726-2730, 1991), polycation compounds such as polylysine, receptor specific ligands, liposomes entrapping the nucleic acid molecules, spheroplast fusion whereby *E. coli* containing the nucleic acid molecules are stripped of their outer cell walls and fused to animal cells using polyethylene glycol, viral transduction, (Cline et al., *Pharmac. Ther. 29*:69, 1985; and Friedmann et al., *Science 244*:1275, 1989), and DNA ligand (Wu et al. *J. of Biol. Chem. 264*:16985-16987, 1989), as well as psoralen inactivated viruses such as Sendai or

Adenovirus. In one embodiment, the rev-caspase construct is introduced into the host cell using a liposome.

As noted above, pharmaceutical compositions also are provided by this invention. These compositions may contain any of the above described inhibitors, enhancers, DNA molecules, vectors or host cells, along with a pharmaceutically or physiologically acceptable carrier, excipients or diluents. Generally, such carriers should be nontoxic to recipients at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining the therapeutic agent with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrins, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with nonspecific serum albumin are exemplary appropriate diluents.

In addition, the pharmaceutical compositions of the present invention may be prepared for administration by a variety of different routes, including for example intraarticularly, intracranially, intradermally, intrahepatically, intramuscularly, intraocularly, intraperitoneally, intrathecally, intravenously, subcutaneously or even directly into a tumor. In addition, pharmaceutical compositions of the present invention may be placed within containers, along with packaging material which provides instructions regarding the use of such pharmaceutical compositions. Generally, such instructions will include a tangible expression describing the reagent concentration, as well as within certain embodiments, relative amounts of excipient ingredients or diluents (*e.g.*, water, saline or PBS) which may be necessary to reconstitute the pharmaceutical composition. Pharmaceutical compositions are useful for both diagnostic or therapeutic purposes.

Pharmaceutical compositions of the present invention may be administered in a manner appropriate to the disease to be treated (or prevented). The quantity and frequency of administration will be determined by such factors as the condition of the patient, and the type and severity of the patient's disease. Dosages may be determined most accurately during clinical trials. Patients may be monitored

for therapeutic effectiveness by appropriate technology, including signs of clinical exacerbation, imaging and the like.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

EXAMPLE 1

GENERATION OF cDNAS EXPRESSING REV-CASPASE-3 AND 6 PRECURSORS

Generation of cDNAs encoding rev-caspase-3 and 6 precursors were generated by PCR. The large (LS) and small subunits (SS) of caspase-3 were amplified with the following primers using the caspase-3 cDNA as a template:

LS-forward, ATGGAGAACACTGAAAACTCAG (SEQ ID NO:43);

LS-reverse, GTCATCATCAACACCTCAGTCT (SEQ ID NO:44);

SS-forward, GGATCCATGATTGAGACAGACAGTGG (SEQ ID NO:45);

SS-reverse, ATCAACTTCATCGTGATAAAAATAGAGTTC (SEQ ID NO:46).

The PCR products were cloned separately into the Sma I site of pBluescript KS$^+$. The small subunit was then excised from KS$^+$-vector with Bam HI and inserted into the Bam HI site of the second KS$^+$-vector which contains the large subunit. This places the small subunit in-frame 5' to the large subunit. Rev-caspase-6 was amplified and cloned in the KS$^+$-vector in a similar way. The following PCR primers were used with caspase-6-His6 cDNA as a template:

LS-forward, ATGAGCTCGGCCTCGGGG (SEQ ID NO:47);

LS'reverse, TTAATCTACTACATCCAAAGG (SEQ ID NO:48);

SS-forward,

GGATCCATGGTAGAAATAGATGCAGCCTCCGTTTAC (SEQ ID NO:49)

SS-reverse, ATCAATTTCAACGTGGTGGTGGTGGTGGTGC (SEQ ID NO:50).

The resulting nucleotide sequences were such that the wild type subunit order was reversed thus creating a contiguous nucleotide sequence wherein the coding region for the small subunit preceded that of the large subunit (See Figure 1). The engineered contiguous caspase-3 and 6 molecules (*i.e.*, rev-caspase molecules) in which the SS was fused in frame N-terminal to the LS, and a cleavage

site (DEVDG in the case of caspase-3; VEIDS in the case of caspase-6 (these internal cleavage sites were designed to be specific for the caspase in which it was introduced in order to investigate the autocatalytic activity of the particular caspase)) was introduced between the two subunits and is depicted in Figures 1B and C.

To express the rev-caspases in bacteria, their cDNAs were excised with Bam HI/Xho I and subcloned into the bacterial expression vector pET28a (Novagen, Inc.) in-frame with the T7-tag of this vector.

EXAMPLE 2

EXPRESSION OF REV-CASPASES IN MAMMALIAN CELLS AND ASSAY FOR APOPTOSIS

To express the rev-caspases in mammalian cells and assay their apoptotic activity, they were amplified with the T7-tag primer and the LS-reverse primers using the pET28a constructs as templates, and subcloned into the mammalian double expression vector pRSC-LacZ (MacFarlane et al., *J. Biol. Chem.*, *272*:25417-25420, 1997; Tsang et al., *Bio/Technology*, *22*:68, 1997). This vector allows the expression of lacZ under the Rous Sarcoma virus promoter, and the test cDNA under the CMV promoter. To assay for apoptosis, MCF-7 or 293 cells were transfected, using the method commercially available as the Lipofect Amine method (Life Technologies, Inc.), with the pRSC-LacZ constructs in the presence or absence of different apoptosis-inhibitors. 30 h after transfection cells were stained with β-galactosidase and examined for morphological signs of apoptosis. The percentage of round blue apoptotic cells (mean ± SD) were represented as a function of total blue cells under each condition ($n \geq 3$).

EXAMPLE 3

IN VITRO TRANSLATION OF CASPASES

^{35}S-labeled caspases (wild-type and rev-caspases) were obtained by *in vitro* translation in the presence of ^{35}S-methionine using a coupled transcription/translation system in rabbit reticulocyte lysate using TNT Kit (Promega) according to the manufacturer's recommendations. Unlike the wild-type caspase-3 and 6, Figure 2A demonstrates that rev-caspase-3 and 6 were able to undergo autocatalytic processing in the *in vitro* translation reaction. Further, this processing was completely inhibited by mutation of the active site Cys of rev-caspase-3 and 6 (Figure 2A, lanes 3 and 6) and by selected caspase inhibitors (See Example 4). Because the *in vitro* translated products are present at very low concentration in the reaction mixture, the observed cleavage must be attributed to an intramolecular processing within the caspase heterotetramer.

EXAMPLE 4

EFFECTS OF INHIBITORS ON REV-CASPASE-3 AND 6 ACTIVITY

To test the effect of selected caspase inhibitors on the autocatalytic activity of rev-caspase-3 and 6, the rev-caspases were translated as in Example 3, but in the presence of varying amounts of inhibitors. As demonstrated by Figure 2B, in the presence of increasing amounts of DEVD-CHO (SEQ ID NO:52), a decrease in the amount of cleavage products and a corresponding increase in the amount of the revcaspase-3 precursor was observed. This corresponded to nearly 50-90% inhibition of the autocatalytic activity of rev-caspase-3 at 40-400 nM concentration. However, the same concentrations of this inhibitor had little effect on the autocatalytic activity of rev-caspase-6 (Figure 2C). This is consistent with earlier observations that caspase-6 is poorly inhibited by DEVD-CHO (SEQ ID NO:52); see Srinivasula et al., *J. Biol. Chem.*, 271:27099-27106, 1996. On the other hand, as is apparent from

inspection of Figures 2B and 2C, z-VAD-fmk had nearly an equal inhibitory effect on rev-caspase-3 and 6 autocatalytic activity at the concentration used in this experiment. Nevertheless, nearly 10-fold more of z-VAD-fmk than DEVD-CHO (SEQ ID NO:52) was required to obtain complete inhibition of caspase-3 activity. Similarly, baculovirus p35 had nearly an equal inhibitory effect on rev-caspase-3 and 6 autocatalytic activity (data not shown).

EXAMPLE 5

CASPASE-3 AND 6 SPECIFICITY RETENTION OF BACTERIALLY EXPRESSED REV-CASPASE-3 AND -6

At limited caspase concentrations, poly(ADP) ribose polymerase (PARP) is specifically cleaved by caspase-3 and 7 but not other caspases. Similarly, lamin is specifically cleaved by caspase-6 but not other caspases. To compare the activity of the wild-type and rev-caspase-3 and 6 towards PARP and lamin, rev-caspase-3 and 6 were expressed in bacteria and then incubated with the two substrates PARP and lamin. As shown in Figures 3A and B, the activity of the rev-caspases towards these two substrates were indistinguishable from their wild-type counterparts. Both caspase-3 variants (rev and WT), but not caspase-6 variants efficiently cleaved PARP. In contrast, both caspase-6 variants, but not caspase-3 variants efficiently cleaved lamin. These results demonstrate that the mature caspases generated from the rev and the wild type constructs have identical substrate specificity.

EXAMPLE 6

INDUCTION OF APOPTOSIS IN MAMMALIAN CELLS BY REV-CASPASE-3 AND -6

To determine the apoptotic activity of rev-caspase-3 and 6 *in vivo*, the rev-caspases were expressed in human MCF-7 cells, transfected as explained above in Example 2. As evidenced by Figures 4A and B, unlike the wild type caspase-3 and 6, the rev-caspases potently induced apoptosis in nearly 90% of the transfected cells. Overexpression of Bc1-2 or CrmA, which protect against different forms of apoptosis, did not significantly reduce their apoptotic activity. Nevertheless, overexpression of the baculovirus p35, which inhibits the activity of most caspases, partially protected against their apoptotic activity. Also, incubation of the transfected cells in the presence of 100 μM z-VAD-fmk, dramatically reduced their apoptotic activity to nearly 30%. These data demonstrate directly that the activity of caspase-3 and 6 are downstream of the CrmA and Bc1-2 block in the apoptotic cascade, and can only be inhibited by high concentration of the pancaspase-inhibitor z-VAD-fmk.

EXAMPLE 7

ACTIVITY OF NONCLEAVABLE REV-CASPASE-3

To demonstrate that the rev-caspase molecules are inherently active and do not require separation of the two subunits and that the two subunits are derived from the same contiguous molecule, the DEVD (SEQ ID NO:52) site was removed and Asp9 and 28, that are present between the two subunits of rev-caspase-3, were mutated (see Figure 2A). However, to follow the activity of this molecule a cleavable 35 residue long His6-T7-tag N-terminal to the IETD (SEQ ID NO:53) site was introduced (see Figure 1B). Figure 5 demonstrates that upon *in vitro* translation of this molecule, as described in Example 3 above, there was no evidence of cleavage between the two subunits. Nevertheless, the translated molecule was active as evident from its ability to cleave its T7-tag to form the p32 species (Figure

5). In the presence of 400 nM DEVD-CHO (SEQ ID NO:52), processing of the T7-tag was inhibited and only the full length p34 species can be seen. Furthermore, expression of this molecule into MCF-7 cells potently induced apoptosis in these cells. These data demonstrate that when the two subunits of a caspase are rearranged in the reverse order, it is not necessary to separate them from each other to generate an active caspase. Thus by mimicking the mature caspase structure, it is possible to design a contiguous active caspase molecules.

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

WE CLAIM:

1. An isolated nucleic acid molecule encoding a rev-caspase.

2. The nucleic acid molecule of claim 1, wherein the rev-caspase is selected from the group consisting of rev-caspase-1, rev-caspase-2, rev-caspase-3, rev-caspase-4, rev-caspase-5, rev-caspase-6, rev-caspase-7, rev-caspase-8, rev-caspase-9, rev-caspase-10, rev-caspase-11, rev-caspase-12, rev-caspase-13, and rev-caspase-14.

3. The nucleic acid molecule of claim 1, wherein the rev-caspase is a human rev-caspase.

4. The nucleic acid molecule of claim 1, wherein the rev-caspase is human rev-caspase-3.

5. The nucleic acid molecule of claim 4, wherein human rev-caspase-3 comprises the sequence recited in Figure 21A (SEQ ID NO:34) or Figure 21B (SEQ ID NO:35) or variants thereof.

6. The nucleic acid molecule of claim 4, wherein human rev-caspase-3 is encoded by the sequence in Figure 7 (SEQ ID NO:1) or Figure 8 (SEQ ID NO:2) or variants thereof.

7. The nucleic acid molecule of claim 1, wherein the rev-caspase is human rev-caspase-6.

8. The nucleic acid molecule of claim 7, wherein human rev-caspase-6 comprises the sequence in Figure 21C (SEQ ID NO:36) or variants thereof.

9. The nucleic acid molecule of claim 7, wherein human rev-caspase-6 is encoded by the sequence in Figure 9 (SEQ ID NO: 3) or variants thereof.

10. An expression vector comprising the nucleic acid molecule of any one of claims 1-9, wherein the sequence encoding rev-caspase is operatively linked to a promoter.

11. The expression vector of claim 10, wherein the promoter is inducible.

12. The expression vector of claim 11, wherein the inducible promoter is HIV LTR.

13. A host cell transfected with the expression vector of claim 10.

14. The host cell of claim 13, wherein the cell is a bacterium or a mammalian cell.

15. A rev-caspase protein.

16. The rev-caspase protein of claim 15, wherein the rev-caspase is selected from the group consisting of rev-caspase-1, rev-caspase-2, rev-caspase-3, rev-caspase-4, rev-caspase-5, rev-caspase-6, rev-caspase-7, rev-caspase-8, rev-caspase-9, rev-caspase-10, rev-caspase-11, rev-caspase-12, rev-caspase-13, and rev-caspase-14.

17. The rev-caspase protein of claim 15, wherein the rev-caspase is a human rev-caspase.

18. The rev-caspase protein of claim 15, wherein the rev-caspase is human rev-caspase-3.

19. The rev-caspase protein of claim 18, wherein human rev-caspase-3 comprises the sequence in Figure 21A (SEQ ID NO:34) or Figure 21B (SEQ ID NO:35) or variants thereof.

20. The rev-caspase protein of claim 18, wherein human rev-caspase-3 is encoded by the sequence in Figure 7 (SEQ ID NO:1) or Figure 8 (SEQ ID NO:2) or variants thereof.

21. The rev-caspase protein of claim 15, wherein the rev-caspase is human rev-caspase-6.

22. The rev-caspase protein of claim 21, wherein human rev-caspase-6 comprises the sequence in Figure 21C (SEQ ID NO:36) or variants thereof.

23. The rev-caspase protein of claim 21, wherein human rev-caspase-6 is encoded by the sequence in Figure 9 (SEQ ID NO:3) or variants thereof.

24. A method of identifying an inhibitor or enhancer of caspase processing activity, comprising:

(a) contacting a sample containing an *in vitro* translated rev-caspase with a candidate inhibitor or candidate enhancer; and

(b) detecting the presence of large and small subunits of rev-caspase, and therefrom determining the level of caspase processing activity, wherein a decrease in processing indicates the presence of a caspase inhibitor, and wherein an increase in processing indicates the presence of a caspase enhancer, wherein processed rev-caspase yields large and small subunits.

25. A method of identifying an inhibitor or enhancer of caspase processing activity, comprising:

(a) contacting a cell transfected with the vector expressing rev-caspase according to claim 10 with a candidate inhibitor or candidate enhancer; and

(b) detecting the presence of large and small subunits of rev-caspase, and therefrom determining the level of caspase processing activity, wherein a decrease in processing indicates the presence of a caspase inhibitor, and wherein an increase in processing indicates the presence of a caspase enhancer, wherein processed rev-caspase yields large and small subunits.

26. A method of identifying an inhibitor or enhancer of caspase-mediated apoptosis, comprising:

(a) contacting a cell transfected with the vector expressing rev-caspase according to claim 10 with a candidate inhibitor or candidate enhancer or with a reference compound; and

(b) detecting cell viability,

wherein viability of cells contacted with a candidate is increased in the presence of an inhibitor and is decreased in the presence of an enhancer compared to cells contacted with a reference compound.

27. The method of any one of claims 24-26, wherein the rev-caspase is selected from the group consisting of rev-caspase-1, rev-caspase-2, rev-caspase-3, rev-caspase-4, rev-caspase-5, rev-caspase-6, rev-caspase-7, rev-caspase-8, rev-caspase-9, rev-caspase-10, rev-caspase-11, rev-caspase-12, rev-caspase-13, and rev-caspase-14.

28. The method of either of claims 24 or 25, wherein detecting comprises gel electrophoresis.

29. The method of claim 24, wherein the *in vitro* translated rev-caspase is labeled.

30. The method of claim 29, wherein the label is a radioactive label, a peptide tag, an enzyme or biotin.

31. A gene delivery vehicle, comprising the nucleic acid molecule according to any one of claims 1-9, wherein the rev-caspase sequence is operatively linked to a promoter.

32. The gene delivery vehicle of claim 31, wherein the vehicle is a retrovirus or adenovirus.

33. The gene delivery vehicle of claim 31, wherein the nucleic acid molecule is associated with a polycation.

34. The gene delivery vehicle of any one of claims 31-33, further comprising a ligand that binds a cell surface receptor.

35. A method of treating cancer, comprising administering to a patient the gene delivery vehicle according to any one of claims 31-34, wherein the gene delivery vehicle is internalized by tumor cells.

36. A method of treating autoimmune disease, comprising administering to a patient the gene delivery vehicle according to any one of claims 31-34, wherein the gene delivery vehicle is internalized by cells mediating autoimmune disease.

Fig. 1A

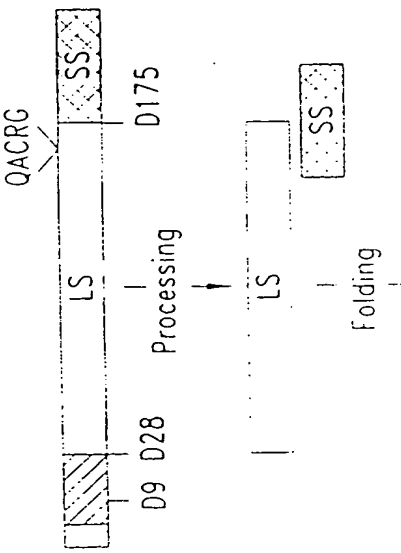


Fig. 1B

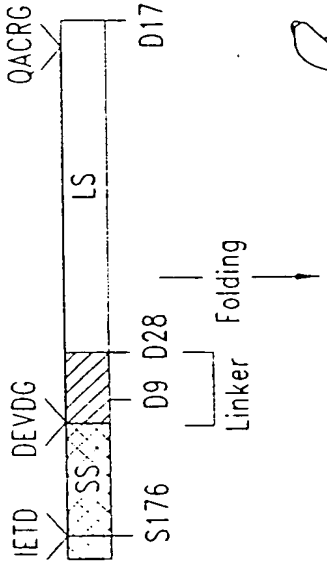
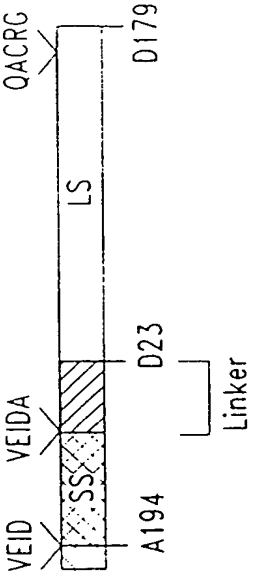
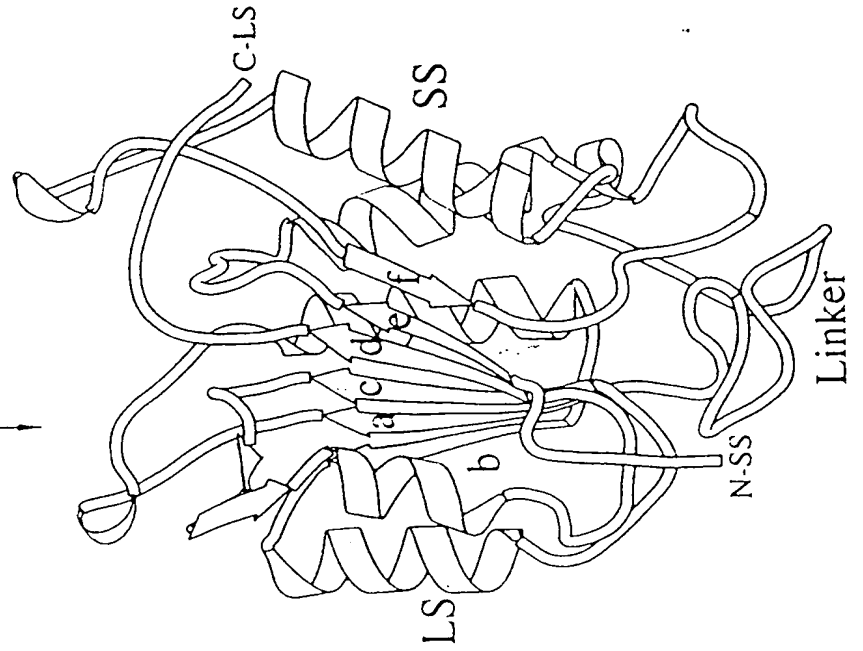
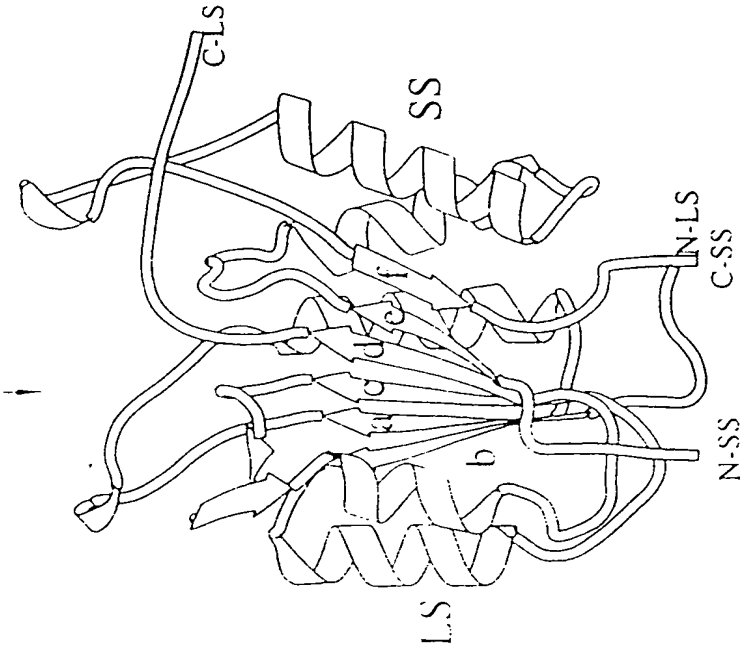
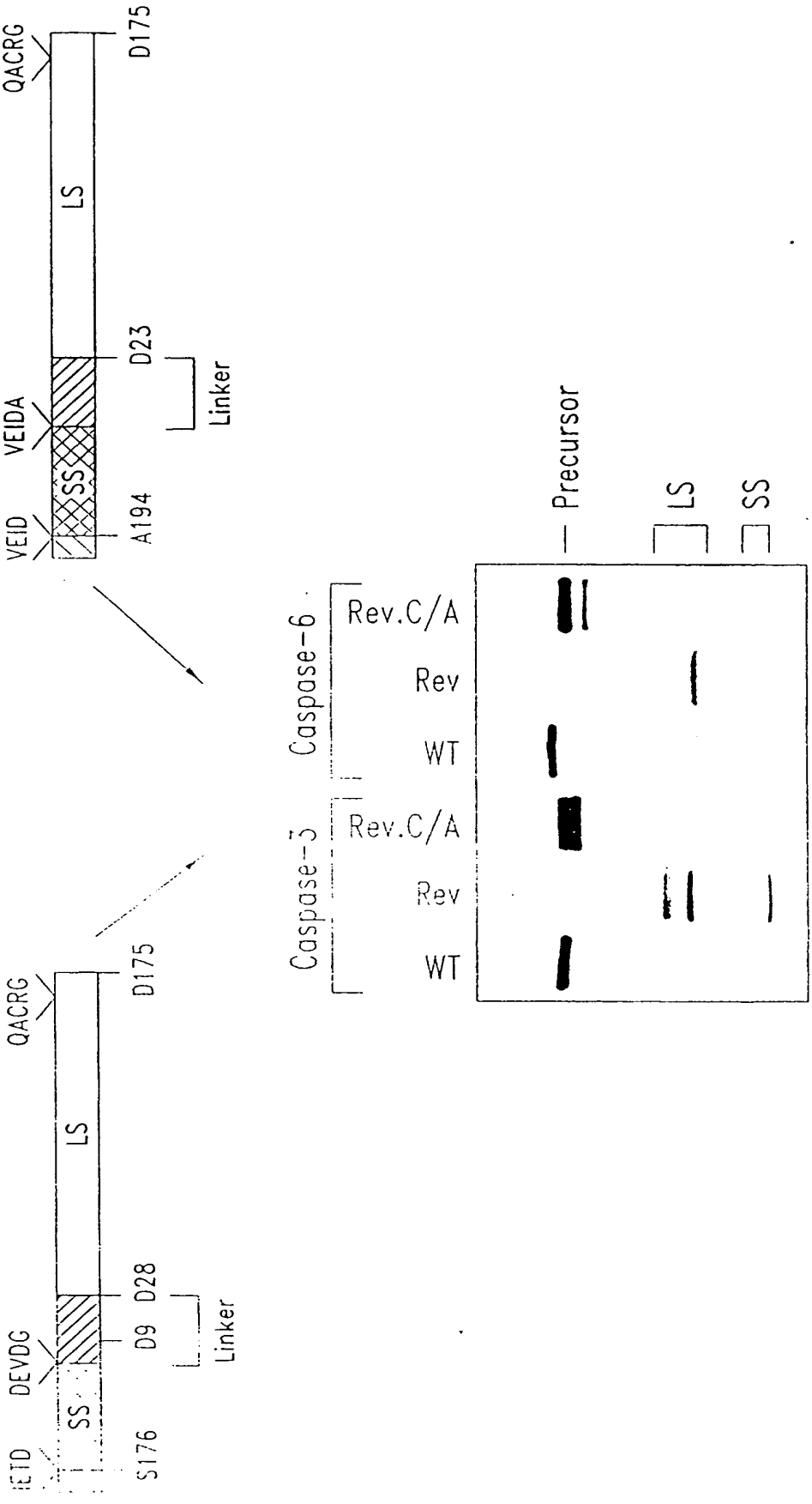


Fig. 1C





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VEIDA
SS
VEID
A194
Precursor
LS
SS
Caspase-6
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Rev
WT
Caspase-3
Rev.C/A
Rev
WT
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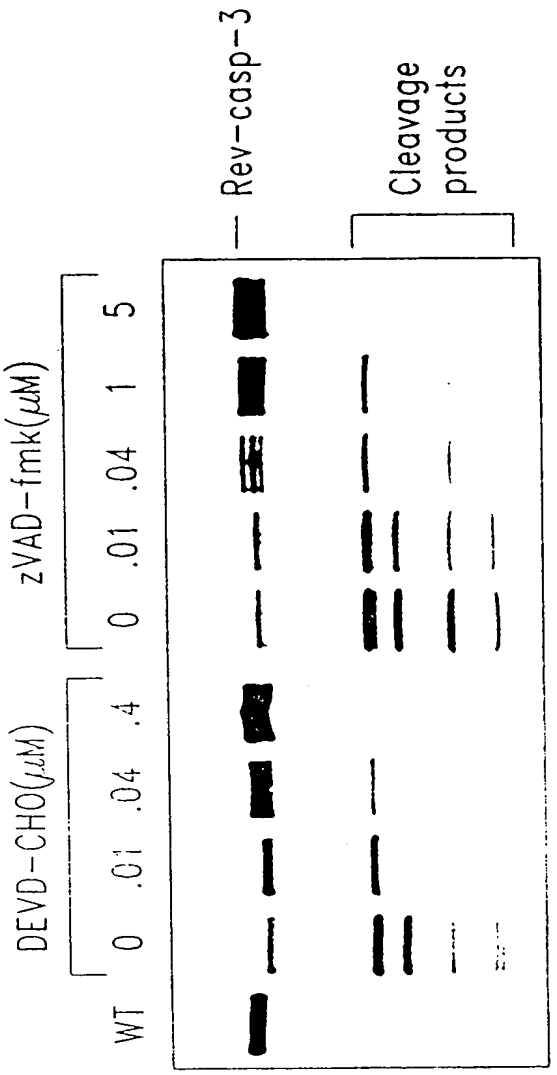
D9
SS
S176

Fig. 2A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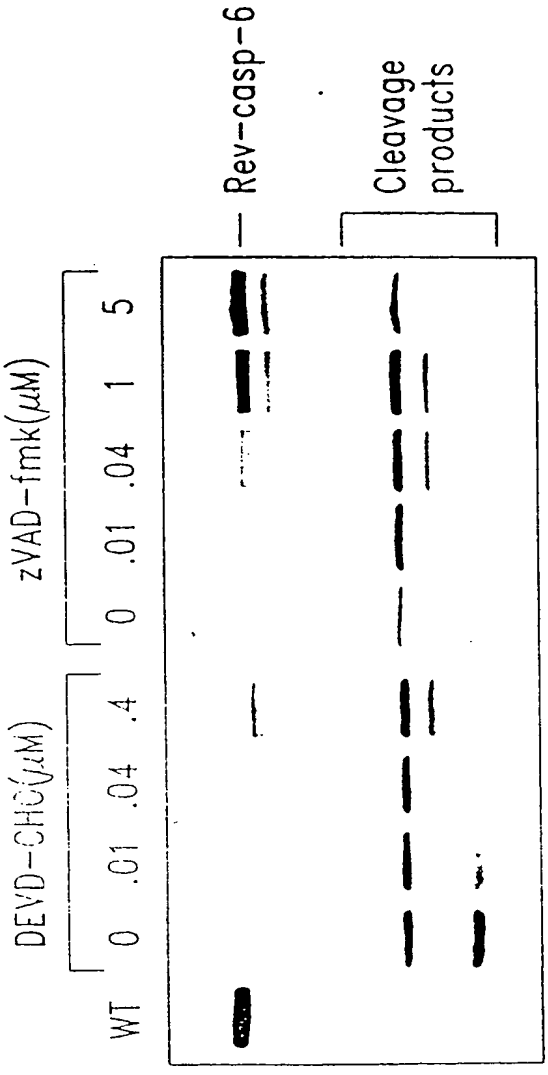
Rev-casp-3
Cleavage products

Fig. 2B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zVAD-fmk(μM)
0 .01 .04 1 5
Rev-casp-6
Cleavage products

Fig. 2C

Fig. 3A

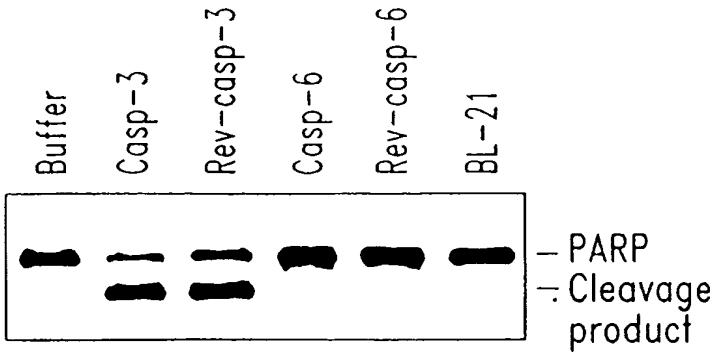


Fig. 3B

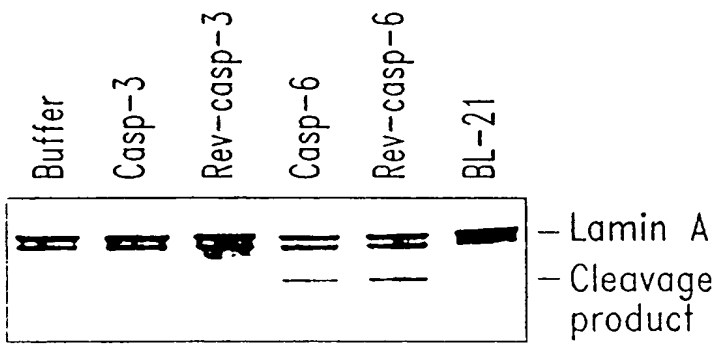


Fig. 4A

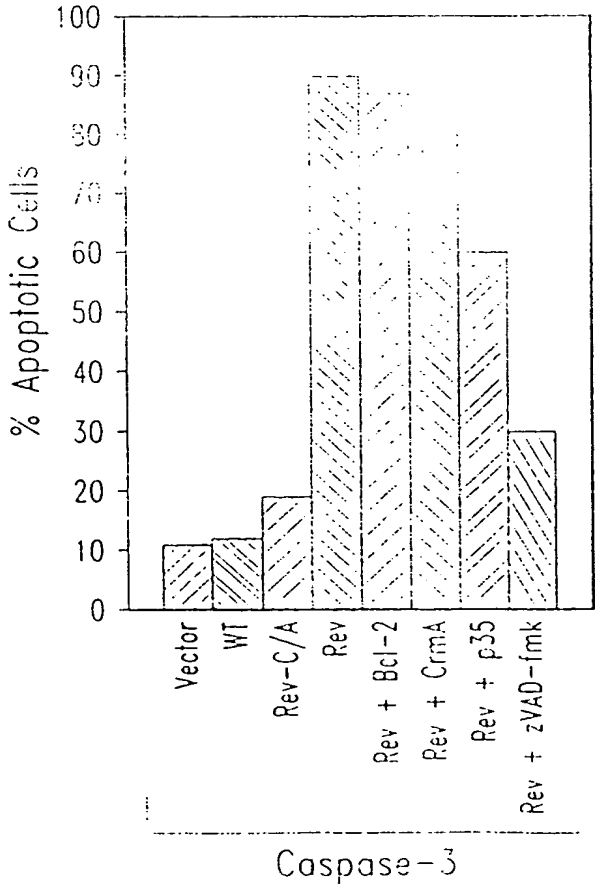
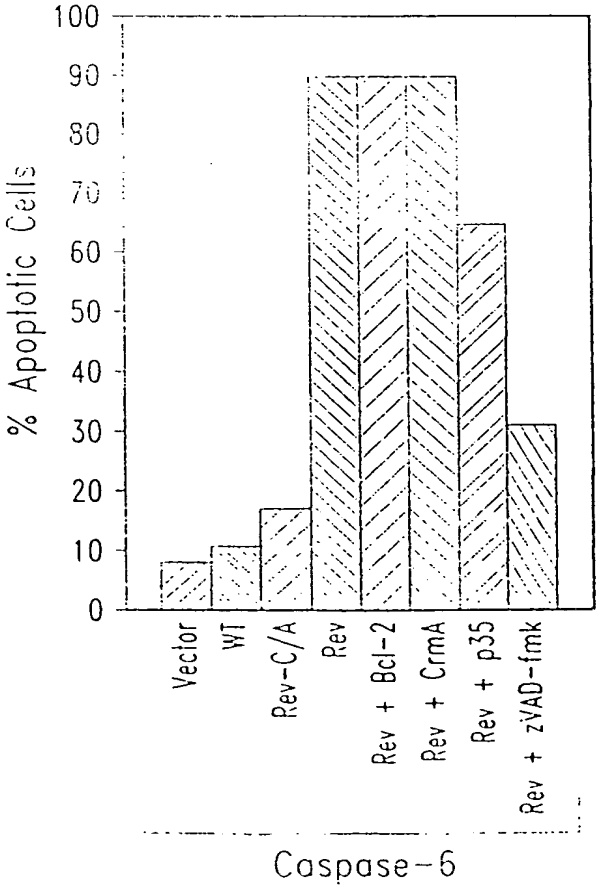
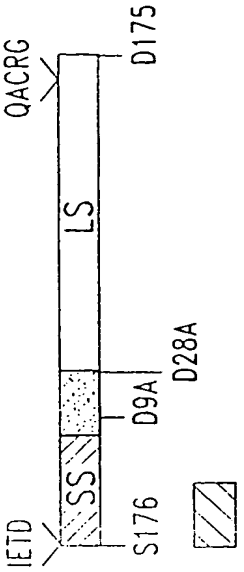


Fig. 4B

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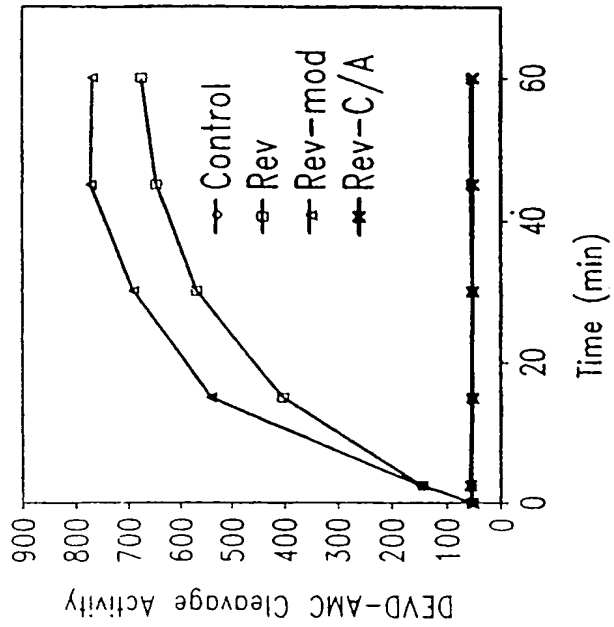
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Rev
Rev-mod
Rev-C/A
DEVD-AMC Cleavage Activity
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300
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500
600
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800
900
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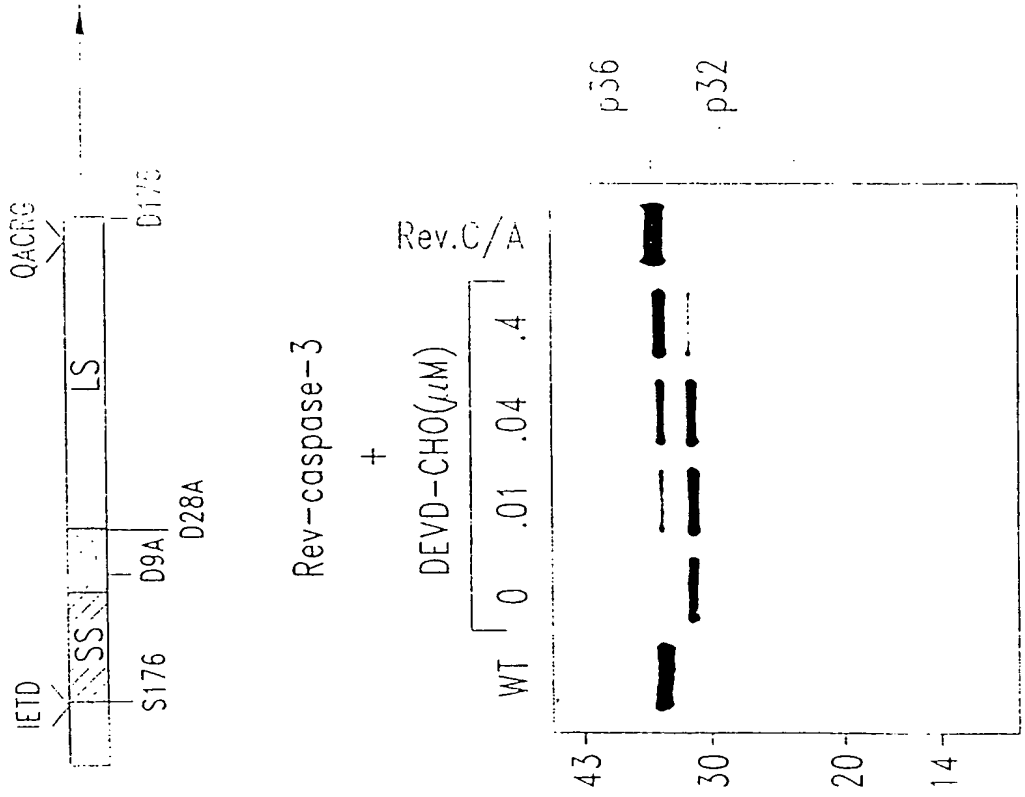
20
40
60

Fig. 5B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Rev-caspase-3
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.04
.4
Rev.C/A
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p32
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30
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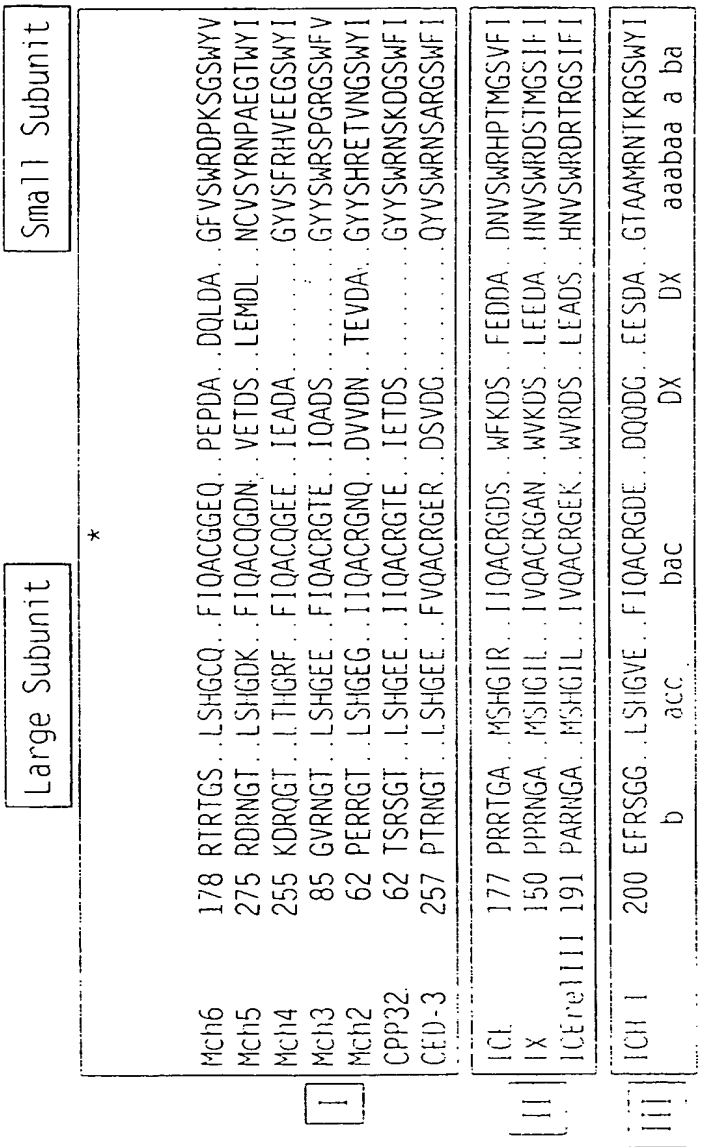
14

Fig. 5A


Large Subunit
Small Subunit
*
I
II
III
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Mch5 275 RDRNGT..LSHGDK..FIQACQGDN..VETDS..LEMDL..NCVSYRNPAEGTWYI
Mch4 255 KDRQGT..LTHGRF..FIQACQGEE..IEADA.....GYVSFRHVEEGSWYI
Mch3 85 GVRNGT..LSHGEE..FIQACRGTE..IQADS.....GYYSWRSPGRGSWFV
Mch2 62 PERRGT..LSHGEG..IIQACRGNQ..DVVDN..TEVDA..GYYSHRETVNGSWYI
CPP32 62 TSRSGT..LSHGEE..IIQACRGTE..IETDS.....GYYSWRNSKDGSWFI
CED-3 257 PTRNGT..LSHGEE..FVQACRGER..DSVDG.....QYVSWRNSARGSWFI
ICL 177 PRRTGA..MSHGIR..IIQACRGDS..WFKDS..FEDDA..DNVSWRHPTMGSVFI
IX 150 PPRNGA..MSHGIL..IVQACRGAN..WVKDS..LEEDA..HNVSWRDSTMGSIFI
ICEre1111 191 PARNGA..MSHGIL..IVQACRGEK..WVRDS..LEADS..HNVSWRDRTRGSIFI
ICH 1 200 EFRSGG..LSHGVE..FIQACRGDE..DQQDG..EESDA..GTAAMRNTKRGSWYI
b acc bac DX DX aaabaa a ba

Fig. 6

Fig. 7

Rev-Caspase-3
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Translation stop 873

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 51 ACCAGTGGAG GCCGACTTCT TGTATGCATA CTCCACAGCA CCTGGTTATT
101 ATTCTTGGCG AAATTCAAAG GATGGCTCCT GGTTCATCCA GTCGCTTTGT
151 GCCATGCTGA AACAGTATGC CGACAAGCTT GAATTTATGC ACATTCTTAC
201 CCGGGTTAAC CGAAAGGTGG CAACAGAATT TGAGTCCTTT TCCTTTGACG
251 CTACTTTTCA TGCAAAGAAA CAGATTCCAT GTATTGTTTC CATGCTCACA
301 AAAGAACTCT ATTTTTATCA CGATGAAGTT GATGGGGGAT CCCCCATGGA
351 GAACACTGAA AACTCAGTGG ATTCAAAATC CATTAAAAAT TTGGAACCAA
401 AGATCATACA TGGAAGCGAA TCAATGGACT CTGGAATATC CCTGGACAAC
451 AGTTATAAAA TGGATTATCC TGAGATGGGT TTATGTATAA TAATTAATAA
501 TAAGAATTTT CATAAGAGCA CTGGAATGAC ATCTCGGTCT GGTACAGATG
551 TCGATGCAGC AAACCTCAGG GAAACATTCA GAAACTTGAA ATATGAAGTC
601 AGGAATAAAA ATGATCTTAC ACGTGAAGAA ATTGTGGAAT TGATGCGTGA
651 TGTTTCTAAA GAAGATCACA GCAAAAGGAG CAGTTTTGTT TGTGTGCTTC
701 TGAGCCATGG TGAAGAAGGA ATAATTTTTG GAACAAATGG ACCTGTTGAC
751 CTGAAAAAAA TAACAAACTT TTTCAGAGGG GATCGTTGTA GAAGTCTAAC
801 TGGAAAACCC AAACTTTTCA TTATTCAGGC CTGCCGTGGT ACAGAACTGG
851 ACTGTGGCAT TGAGACAGAC TGA
```

Fig. 8

Uncleavable Rev-caspase-3
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101 ATTCTTGGCG AAATTCAAAG GATGGCTCCT GGTTCATCCA GTCGCTTTGT
151 GCCATGCTGA AACAGTATGC CGACAAGCTT GAATTTATGC ACATTCTTAC
201 CCGGGTTAAC CGAAAGGTGG CAACAGAATT TGAGTCCTTT TCCTTTGACG
251 CTACTTTTCA TGCAAAGAAA CAGATTCCAT GTATTGTTTC CATGCTCACA
301 AAAGAACTCT ATTTTTATCA CGGATCCCCC ATGGAGAACA CTGAAAACTC
351 AGTGGCTTCA AAATCCATTA AAAATTTGGA ACCAAAGATC ATACATGGAA
401 GCGAATCAAT GGCCTCTGGA ATATCCCTGG ACAACAGTTA TAAAATGGAT
451 TATCCTGAGA TGGGTTTATG TATAATAATT AATAATAAGA ATTTTCATAA
501 GAGCACTGGA ATGACATCTC GGTCTGGTAC AGATGTCGAT GCAGCAAACC
551 TCAGGGAAAC ATTCAGAAAC TTGAAATATG AAGTCAGGAA TAAAAATGAT
601 CTTACACGTG AAGAAATTGT GGAATTGATG CGTGATGTTT CTAAAGAAGA
651 TCACAGCAAA AGGAGCAGTT TTGTTTGTGT GCTTCTGAGC CATGGTGAAG
701 AAGGAATAAT TTTTGGAACA AATGGACCTG TTGACCTGAA AAAAATAACA
751 AACTTTTTCA GAGGGGATCG TTGTAGAAGT CTAACTGGAA AACCCAAACT
801 TTTCATTATT CAGGCCTGCC GTGGTACAGA ACTGGACTGT GGCATTGAGA
851 CAGACTGA
```

Fig. 9

Rev-caspase-6
Translation start 1
Translation stop 903

```
  1 ATGGTAGAAA TAGATGCAGC CTCCGTTTAC ACGCTGCCTG CTGGAGCTGA
 51 CTTCCTCATG TGTTACTCTG TTGCAGAAGG ATATTATTCT CACCGGGAAA
101 CTGTGAACGG CTCATGGTAC ATTCAAGATT TGTGTGAGAT GTTGGGAAAA
151 TATGGCTCCT CCTTAGAGTT CACAGAACTC CTCACACTGG TGAACAGGAA
201 AGTTTCTCAG CGCCGAGTGG ACTTTTGCAA AGACCCAAGT GCAATTGGAA
251 AGAAGCAGGT TCCCTGTTTT GCCTCAATGC TAACTAAAAA GCTGCATTTC
301 TTTCCAAAAT CTAATCTCGA GCACCACCAC CACCACCACG TTGAAATTGA
351 TGGGGGATCC CCCATGAGCT CGGCCTCGGG GCTCCGCAGG GGGCACCCGG
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651 ACTGCTCAAA ATTCATGAGG TGTCAACTGT TAGCCACGCA GATGCCGATT
701 GCTTTGTGTG TGTCTTCCTG AGCCATGGCG AAGGCAATCA CATTTATGCA
751 TATGATGCTA AAATCGAAAT TCAGACATTA ACTGGCTTGT TCAAAGGAGA
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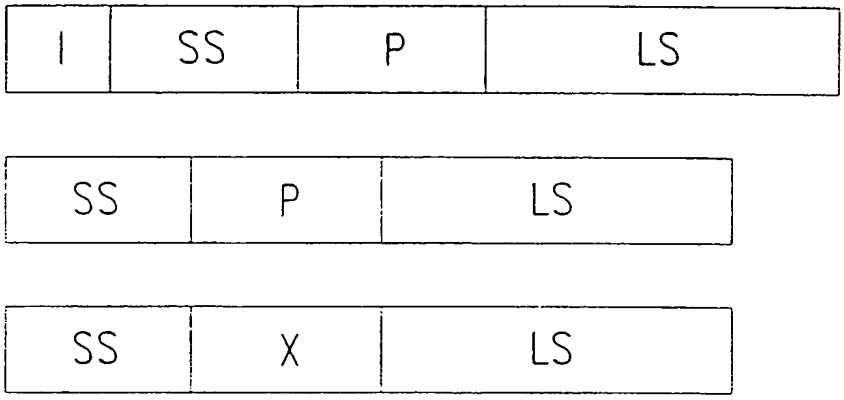

Fig. 10

Fig. 11A

CASPASE 1

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    TACCGGCTGTTCCAGGACTTCCTCTTCTCTTTCGACAAATAGGCAAGGTACCCACTTCCA
----------------------------------------------------------------
+1  ThrIleAsnGlyLeuLeuAspGluLeuLeuGlnThrArgValLeuAsnLysGluGluMet
    ----------------------------------------------------------
61  ACAATAAATGGCTTACTGGATGAATTATTACAGACAAGGGTGCTGAACAAGGAAGAGATG
    TGTTATTTACCGAATGACCTACTTAATAATGTCTGTTCCCACGACTTGTTCCTTCTCTAC
----------------------------------------------------------------
+1  GluLysValLysArgGluAsnAlaThrValMetAspLysThrArgAlaLeuIleAspSer
    ----------------------------------------------------------
121 GAGAAAGTAAAACGTGAAAATGCTACAGTTATGGATAAGACCCGAGCTTTGATTGACTCC
    CTCTTTCATTTTGCACTTTTACGATGTCAATACCTATTCTGGGCTCGAAACTAACTGAGG
----------------------------------------------------------------
+1  ValIleProLysGlyAlaGlnAlaCysGlnIleCysIleThrTyrIleCysGluGluAsp
    ----------------------------------------------------------
181 GTTATTCCGAAAGGGGCACAGGCATGCCAAATTTGCATCACATACATTTGTGAAGAAGAC
    CAATAAGGCTTTCCCCGTGTCCGTACGGTTTAAACGTAGTGTATGTAAACACTTCTTCTG
----------------------------------------------------------------
+1  SerTyrLeuAlaGlyThrLeuGlyLeuSerAlaAspGlnThrSerGlyAsnTyrLeuAsn
    ----------------------------------------------------------
241 AGTTACCTGGCAGGGACGCTGGGACTCTCAGCAGATCAAACATCTGGAAATTACCTTAAT
    TCAATGGACCGTCCCTGCGACCCTGAGAGTCGTCTAGTTTGTAGACCTTTAATGGAATTA
----------------------------------------------------------------
+1  MetGlnAspSerGlnGlyValLeuSerSerPheProAlaProGlnAlaValGlnAspAsn
    ----------------------------------------------------------
301 ATGCAAGACTCTCAAGGAGTACTTTCTTCCTTTCCAGCTCCTCAGGCAGTGCAGGACAAC
    TACGTTCTGAGAGTTCCTCATGAAAGAAGGAAAGGTCGAGGAGTCCGTCACGTCCTGTTG
----------------------------------------------------------------
+1  ProAlaMetProThrSerSerGlySerGluGlyAsnValLysLeuCysSerLeuGluGlu
    ----------------------------------------------------------
361 CCAGCTATGCCCACATCCTCAGGCTCAGAAGGGAATGTCAAGCTTTGCTCCCTAGAAGAA
    GGTCGATACGGGTGTAGGAGTCCGAGTCTTCCCTTACAGTTCGAAACGAGGGATCTTCTT
----------------------------------------------------------------
+1  AlaGlnArgIleTrpLysGlnLysSerAlaGluIleTyrProIleMetAspLysSerSer
    ----------------------------------------------------------
421 GCTCAAAGGATATGGAAACAAAAGTCGGCAGAGATTTATCCAATAATGGACAAGTCAAGC
    CGAGTTTCCTATACCTTTGTTTTCAGCCGTCTCTAAATAGGTTATTACCTGTTCAGTTCG
----------------------------------------------------------------
+1  ArgThrArgLeuAlaLeuIleIleCysAsnGluGluPheAspSerIleProArgArgThr
    ----------------------------------------------------------
481 CGCACACGTCTTGCTCTCATTATCTGCAATGAAGAATTTGACAGTATTCCTAGAAGAACT
    GCGTGTGCAGAACGAGAGTAATAGACGTTACTTCTTAAACTGTCATAAGGATCTTCTTGA
----------------------------------------------------------------
+1  GlyAlaGluValAspIleThrGlyMetThrMetLeuLeuGlnAsnLeuGlyTyrSerVal
    ----------------------------------------------------------
541 GGAGCTGAGGTTGACATCACAGGCATGACAATGCTGCTACAAAATCTGGGGTACAGCGTA
    CCTCGACTCCAACTGTAGTGTCCGTACTGTTACGACGATGTTTTAGACCCCATGTCGCAT
----------------------------------------------------------------
+1  AspValLysLysAsnLeuThrAlaSerAspMetThrThrGluLeuGluAlaPheAlaHis
    ----------------------------------------------------------
601 GATGTGAAAAAAAATCTCACTGCTTCGGACATGACTACAGAGCTGGAGGCATTTGCACAC
    CTACACTTTTTTTTAGAGTGACGAAGCCTGTACTGATGTCTCGAACCTCCGTAAACGTGTG
```

Fig. 11B

CASPASE 1

```
  +1 ArgProGluHisLysThrSerAspSerThrPheLeuValPheMetSerHisGlyIleArg
     ------------------------------------------------------------
 661 CGCCCAGAGCACAAGACCTCTGACAGCACGTTCCTGGTGTTCATGTCTCATGGTATTCGG
     GCGGGTCTCGTGTTCTGGAGACTGTCGTGCAAGGACCACAAGTACAGAGTACCATAAGCC
  +1 GluGlyIleCysGlyLysLysHisSerGluGlnValProAspIleLeuGlnLeuAsnAla
     ------------------------------------------------------------
 721 GAAGGCATTTGTGGGAAGAAACACTCTGAGCAAGTCCCAGATATACTACAACTCAATGCA
     CTTCCGTAAACACCCTTCTTTGTGAGACTCGTTCAGGGTCTATATGATGTTGAGTTACGT
  +1 IlePheAsnMetLeuAsnThrLysAsnCysProSerLeuLysAspLysProLysValIle
     ------------------------------------------------------------
 781 ATCTTTAACATGTTGAATACCAAGAACTGCCCAAGTTTGAAGGACAAACCGAAGGTGATC
     TAGAAATTGTACAACTTATGGTTCTTGACGGGTTCAAACTTCCTGTTTGGCTTCCACTAG
  +1 IleIleGlnAlaCysArgGlyAspSerProGlyValValTrpPheLysAspSerValGly
     ------------------------------------------------------------
 841 ATCATCCAGGCCTGCCGTGGTGACAGCCCTGGTGTGGTGTGGTTTAAAGATTCAGTAGGA
     TAGTAGGTCCGGACGGCACCACTGTCGGGACCACACCACACCAAATTTCTAAGTCATCCT
  +1 ValSerGlyAsnLeuSerLeuProThrThrGluGluPheGluAspAspAlaIleLysLys
     ------------------------------------------------------------
 901 GTTTCTGGAAACCTATCTTTACCAACTACAGAAGAGTTTGAGGATGATGCTATTAAGAAA
     CAAAGACCTTTGGATAGAAATGGTTGATGTCTTCTCAAACTCCTACTACGATAATTCTTT
  +1 AlaHisIleGluLysAspPheIleAlaPheCysSerSerThrProAspAsnValSerTrp
     ------------------------------------------------------------
 961 GCCCACATAGAGAAGGATTTTATCGCTTTCTGCTCTTCCACACCAGATAATGTTTCTTGG
     CGGGTGTATCTCTTCCTAAAATAGCGAAAGACGAGAAGGTGTGGTCTATTACAAAGAACC
  +1 ArgHisProThrMetGlySerValPheIleGlyArgLeuIleGluHisMetGlnGluTyr
     ------------------------------------------------------------
1021 AGACATCCCACAATGGGCTCTGTTTTTATTGGAAGACTCATTGAACATATGCAAGAATAT
     TCTGTAGGGTGTTACCCGAGACAAAAATAACCTTCTGAGTAACTTGTATACGTTCTTATA
  +1 AlaCysSerCysAspValGluGluIlePheArgLysValArgPheSerPheGluGlnPro
     ------------------------------------------------------------
1081 GCCTGTTCCTGTGATGTGGAGGAAATTTTCCGCAAGGTTCGATTTTCATTTGAGCAGCCA
     CGGACAAGGACACTACACCTCCTTTAAAAGGCGTTCCAAGCTAAAAGTAAACTCGTCGGT
  +1 AspGlyArgAlaGlnMetProThrThrGluArgValThrLeuThrArgCysPheTyrLeu
     ------------------------------------------------------------
1141 GATGGTAGAGCGCAGATGCCCACCACTGAAAGAGTGACTTTGACAAGATGTTTCTACCTC
     CTACCATCTCGCGTCTACGGGTGGTGACTTTCTCACTGAAACTGTTCTACAAAGATGGAG
  +1 PheProGlyHis
     ---------->
1201 TTCCCAGGACATTAA
     AAGGGTCCTGTAATT
```

Fig. 12A

CASPASE 2

Fig. 12B

CASPASE 2

```
  +1 TyrAspValHisValLeuCysAspGlnThrAlaGlnGluMetGlnGluLysLeuGlnAsn
     ------------------------------------------------------------
661  TATGACGTCCATGTTCTATGTGACCAGACTGCACAGGAAATGCAAGAGAAACTGCAGAAT
     ATACTGCAGGTACAAGATACACTGGTCTGACGTGTCCTTTACGTTCTCTTTGACGTCTTA
  +1 PheAlaGlnLeuProAlaHisArgValThrAspSerCysIleValAlaLeuLeuSerHis
     ------------------------------------------------------------
721  TTTGCACAGTTACCTGCACACCGAGTCACGGACTCCTGCATCGTGGCACTCCTCTCGCAT
     AAACGTGTCAATGGACGTGTGGCTCAGTGCCTGAGGACGTAGCACCGTGAGGAGAGCGTA
  +1 GlyValGluGlyAlaIleTyrGlyValAspGlyLysLeuLeuGlnLeuGlnGluValPhe
     ------------------------------------------------------------
781  GGTGTGGAGGGCGCCATCTATGGTGTGGATGGGAAACTGCTCCAGCTCCAAGAGGTTTTT
     CCACACCTCCCGCGGTAGATACCACACCTACCCTTTGACGAGGTCGAGGTTCTCCAAAAA
  +1 GlnLeuPheAspAsnAlaAsnCysProSerLeuGlnAsnLysProLysMetPhePheIle
     ------------------------------------------------------------
841  CAGCTCTTTGACAACGCCAACTGCCCAAGCCTACAGAACAAACCAAAAATGTTCTTCATC
     GTCGAGAAACTGTTGCGGTTGACGGGTTCGGATGTCTTGTTTGGTTTTTACAAGAAGTAG
  +1 GlnAlaCysArgGlyAspGluThrAspArgGlyValAspGlnGlnAspGlyLysAsnHis
     ------------------------------------------------------------
901  CAGGCCTGCCGTGGAGATGAGACTGATCGTGGGGTTGACCAACAAGATGGAAAGAACCAC
     GTCCGGACGGCACCTCTACTCTGACTAGCACCCCAACTGGTTGTTCTACCTTTCTTGGTG
  +1 AlaGlySerProGlyCysGluGluSerAspAlaGlyLysGluLysLeuProLysMetArg
     ------------------------------------------------------------
961  GCAGGATCCCCTGGGTGCGAGGAGAGTGATGCCGGTAAAGAAAAGTTGCCGAAGATGAGA
     CGTCCTAGGGGACCCACGCTCCTCTCACTACGGCCATTTCTTTTCAACGGCTTCTACTCT
  +1 LeuProThrArgSerAspMetIleCysGlyTyrAlaCysLeuLysGlyThrAlaAlaMet
     ------------------------------------------------------------
1021 CTGCCCACGCGCTCAGACATGATATGCGGCTATGCCTGCCTCAAAGGGACTGCCGCCATG
     GACGGGTGCGCGAGTCTGTACTATACGCCGATACGGACGGAGTTTCCCTGACGGCGGTAC
  +1 ArgAsnThrLysArgGlySerTrpTyrIleGluAlaLeuAlaGlnValPheSerGluArg
     ------------------------------------------------------------
1081 CGGAACACCAAACGAGGTTCCTGGTACATCGAGGCTCTTGCTCAAGTGTTTTCTGAGCGG
     GCCTTGTGGTTTGCTCCAAGGACCATGTAGCTCCGAGAACGAGTTCACAAAAGACTCGCC
  +1 AlaCysAspMetHisValAlaAspMetLeuValLysValAsnAlaLeuIleLysAspArg
     ------------------------------------------------------------
1141 GCTTGTGATATGCACGTGGCCGACATGCTGGTTAAGGTGAACGCACTTATCAAGGATCGG
     CGAACACTATACGTGCACCGGCTGTACGACCAATTCCACTTGCGTGAATAGTTCCTAGCC
  +1 GluGlyTyrAlaProGlyThrGluPheHisArgCysLysGluMetSerGluTyrCysSer
     ------------------------------------------------------------
1201 GAAGGTTATGCTCCTGGCACAGAATTCCACCGGTGCAAGGAAATGTCTGAATACTGCAGC
     CTTCCAATACGAGGACCGTGTCTTAAGGTGGCCACGTTCCTTTACAGACTTATGACGTCG
  +1 ThrLeuCysArgHisLeuTyrLeuPheProGlyHisProProThr
     ---------------------------------------------->
1261 ACTCTGTGCCGCCACCTCTACCTGTTCCCAGGACACCCTCCCACATGA
     TGAGACACGGCGGTGGAGATGGACAAGGGTCCTGTGGGAGGGTGTACT
```

Fig. 13A

CASPASE 3

Fig. 13B

CASPASE 3

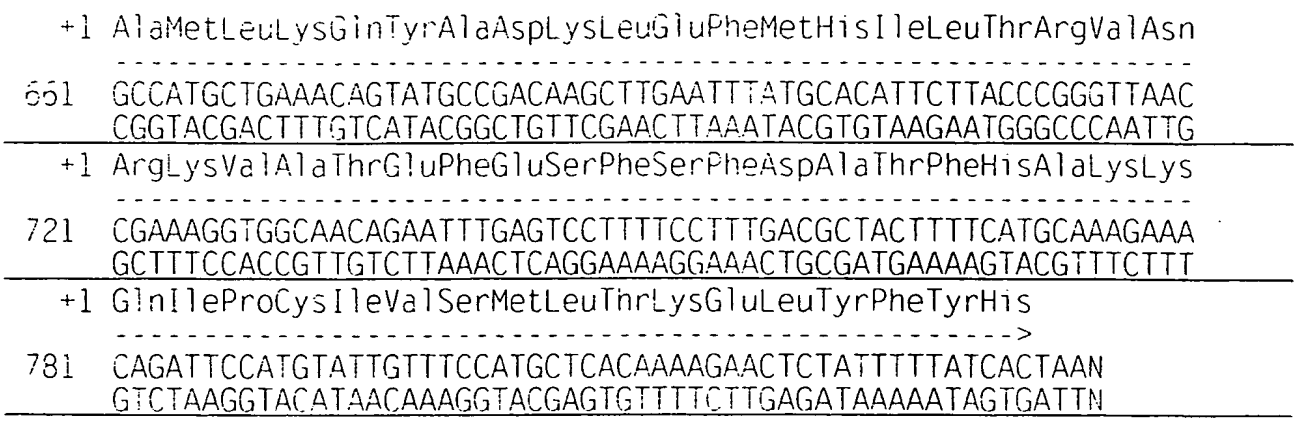

Fig. 14A

CASPASE 4

Fig. 14B

CASPASE 4

```
   +1 HisAspGluLysLysProAspValLeuLeuTyrAspThrIlePheGlnIlePheAsnAsn
      ------------------------------------------------------------
661   CATGATGAGAAAAAACCAGATGTGCTGCTTTATGACACCATCTTCCAGATATTCAACAAC
      GTACTACTCTTTTTTGGTCTACACGACGAAATACTGTGGTAGAAGGTCTATAAGTTGTTG
   +1 ArgAsnCysLeuSerLeuLysAspLysProLysValIleIleValGlnAlaCysArgGly
      ------------------------------------------------------------
721   CGCAACTGCCTCAGTCTGAAGGACAAACCCAAGGTCATCATTGTCCAGGCCTGCAGAGGT
      GCGTTGACGGAGTCAGACTTCCTGTTTGGGTTCCAGTAGTAACAGGTCCGGACGTCTCCA
   +1 AlaAsnArgGlyGluLeuTrpValArgAspSerProAlaSerLeuGluValAlaSerSer
      ------------------------------------------------------------
781   GCAAACCGTGGGGAACTGTGGGTCAGAGACTCTCCAGCATCCTTGGAAGTGGCCTCTTCA
      CGTTTGGCACCCCTTGACACCCAGTCTCTGAGAGGTCGTAGGAACCTTCACCGGAGAAGT
   +1 GlnSerSerGluAsnLeuGluGluAspAlaValTyrLysThrHisValGluLysAspPhe
      ------------------------------------------------------------
841   CAGTCATCTGAGAACCTGGAGGAAGATGCTGTTTACAAGACCCACGTGGAGAAGGACTTC
      GTCAGTAGACTCTTGGACCTCCTTCTACGACAAATGTTCTGGGTGCACCTCTTCCTGAAG
   +1 IleAlaPheCysSerSerThrProHisAsnValSerTrpArgAspSerThrMetGlySer
      ------------------------------------------------------------
901   ATTGCTTTCTGCTCTTCAACGCCACACAACGTGTCCTGGAGAGACAGCACAATGGGCTCT
      TAACGAAAGACGAGAAGTTGCGGTGTGTTGCACAGGACCTCTCTGTCGTGTTACCCGAGA
   +1 IlePheIleThrGlnLeuIleThrCysPheGlnLysTyrSerTrpCysCysHisLeuGlu
      ------------------------------------------------------------
961   ATCTTCATCACACAACTCATCACATGCTTCCAGAAATATTCTTGGTGCTGCCACCTAGAG
      TAGAAGTAGTGTGTTGAGTAGTGTACGAAGGTCTTTATAAGAACCACGACGGTGGATCTC
   +1 GluValPheArgLysValGlnGlnSerPheGluThrProArgAlaLysAlaGlnMetPro
      ------------------------------------------------------------
1021  GAAGTATTTCGGAAGGTACAGCAATCATTTGAAACTCCAAGGGCCAAAGCTCAAATGCCC
      CTTCATAAAGCCTTCCATGTCGTTAGTAAACTTTGAGGTTCCCGGTTTCGAGTTTACGGG
   +1 ThrIleGluArgLeuSerMetThrArgTyrPheTyrLeuPheProGlyAsn
      ------------------------------------------------->
1081  ACCATAGAACGACTGTCCATGACAAGATATTTCTACCTCTTTCCTGGCAATTGA
      TGGTATCTTGCTGACAGGTACTGTTCTATAAAGATGGAGAAAGGACCGTTAACT
```

Fig. 15A

CASPASE 5

Fig. 15B

CASPASE 5

```
  -1 ThrAlaArgAspMetGluSerValLeuArgAlaPheAlaAlaArgProGluHisLysSer
     ------------------------------------------------------------
 661 ACAGCCAGGGATATGGAGTCAGTGCTGAGGGCATTTGCTGCCAGACCAGAGCACAAGTCC
     TGTCGGTCCCTATACCTCAGTCACGACTCCCGTAAACGACGGTCTGGTCTCGTGTTCAGG
  -1 SerAspSerThrPheLeuValLeuMetSerHisGlyIleLeuGluGlyIleCysGlyThr
     ------------------------------------------------------------
 721 TCTGACAGCACGTTCTTGGTACTCATGTCTCATGGCATCCTAGAGGGAATCTGCGGAACT
     AGACTGTCGTGCAAGAACCATGAGTACAGAGTACCGTAGGATCTCCCTTAGACGCCTTGA
  +1 AlaHisLysLysLysLysProAspValLeuLeuTyrAspThrIlePheGlnIlePheAsn
     ------------------------------------------------------------
 781 GCGCATAAAAAGAAAAAACCGGATGTGCTGCTTTATGACACCATCTTCCAGATATTCAAC
     CGCGTATTTTTCTTTTTTGGCCTACACGACGAAATACTGTGGTAGAAGGTCTATAAGTTG
  -1 AsnArgAsnCysLeuSerLeuLysAspLysProLysValIleIleValGlnAlaCysArg
     ------------------------------------------------------------
 841 AACCGCAACTGCCTCAGTCTAAAGGACAAACCCAAGGTCATCATTGTCCAGGCCTGCAGA
     TTGGCGTTGACGGAGTCAGATTTCCTGTTTGGGTTCCAGTAGTAACAGGTCCGGACGTCT
  +1 GlyGluLysHisGlyGluLeuTrpValArgAspSerProAlaSerLeuAlaValIleSer
     ------------------------------------------------------------
 901 GGTGAAAAACATGGGGAACTCTGGGTCAGAGACTCTCCAGCATCCTTGGCAGTCATCTCT
     CCACTTTTTGTACCCCTTGAGACCCAGTCTCTGAGAGGTCGTAGGAACCGTCAGTAGAGA
  +1 SerGlnSerSerGluAsnLeuGluAlaAspSerValCysLysIleHisGluGluLysAsp
     ------------------------------------------------------------
 961 TCACAGTCATCTGAGAACCTGGAGGCAGATTCTGTTTGCAAGATCCACGAGGAGAAGGAC
     AGTGTCAGTAGACTCTTGGACCTCCGTCTAAGACAAACGTTCTAGGTGCTCCTCTTCCTG
  +1 PheIleAlaPheCysSerSerThrProHisAsnValSerTrpArgAspArgThrArgGly
     ------------------------------------------------------------
1021 TTCATTGCTTTCTGTTCTTCAACACCACATAACGTGTCCTGGAGAGACCGCACAAGGGGC
     AAGTAACGAAAGACAAGAAGTTGTGGTGTATTGCACAGGACCTCTCTGGCGTGTTCCCCG
  +1 SerIlePheIleThrGluLeuIleThrCysPheGlnLysTyrSerCysCysCysHisLeu
     ------------------------------------------------------------
1081 TCCATCTTCATTACGGAACTCATCACATGCTTCCAGAAATATTCTTGCTGCTGCCACCTA
     AGGTAGAAGTAATGCCTTGAGTAGTGTACGAAGGTCTTTATAAGAACGACGACGGTGGAT
  +1 MetGluIlePheArgLysValGlnLysSerPheGluValProGlnAlaLysAlaGlnMet
     ------------------------------------------------------------
1141 ATGGAAATATTTCGGAAGGTACAGAAATCATTTGAAGTTCCACAGGCTAAAGCCCAGATG
     TACCTTTATAAAGCCTTCCATGTCTTTAGTAAACTTCAAGGTGTCCGATTTCGGGTCTAC
  -1 ProThrIleGluArgAlaThrLeuThrArgAspPheTyrLeuPheProGlyAsn
     ------------------------------------------------------>
1201 CCCACCATAGAACGAGCAACGTTGACAAGAGATTTCTACCTCTTTCCTGGCAATTGAN
     GGGTGGTATCTTGCTCGTTGGAACTGTTCTCTAAAGATGGAGAAAGGACCGTTAACTN
```

Fig. 16A

CASPASE 6

```
   +1 MetSerSerAlaSerGlyLeuArgArgGlyHisProAlaGlyGlyGluGluAsnMetThr
      ]----------------------------------------------------------
    1 ATGAGCTCGGCCTCGGGGCTCCGCAGGGGGCACCCGGCAGGTGGGGAAGAAAACATGACA
      TACTCGAGCCGGAGCCCCGAGGCGTCCCCCGTGGGCCGTCCACCCCTTCTTTTGTACTGT
   +1 GluThrAspAlaPheTyrLysArgGluMetPheAspProAlaGluLysTyrLysMetAsp
      ----------------------------------------------------------
   61 GAAACAGATGCCTTCTATAAAAGAGAAATGTTTGATCCGGCAGAAAAGTACAAAATGGAC
      CTTTGTCTACGGAAGATATTTTCTCTTTACAAACTAGGCCGTCTTTTCATGTTTTACCTG
   +1 HisArgArgArgGlyIleAlaLeuIlePheAsnHisGluArgPhePheTrpHisLeuThr
      ----------------------------------------------------------
  121 CACAGGAGGAGAGGAATTGCTTTAATCTTCAATCATGAGAGGTTCTTTTGGCACTTAACA
      GTGTCCTCCTCTCCTTAACGAAATTAGAAGTTAGTACTCTCCAAGAAAACCGTGAATTGT
   +1 LeuProGluArgArgGlyThrCysAlaAspArgAspAsnLeuThrArgArgPheSerAsp
      ----------------------------------------------------------
  181 CTGCCAGAAAGGCGGGGCACCTGCGCAGATAGAGACAATCTTACCCGCAGGTTTTCAGAT
      GACGGTCTTTCCGCCCCGTGGACGCGTCTATCTCTGTTAGAATGGGCGTCCAAAAGTCTA
   +1 LeuGlyPheGluValLysCysPheAsnAspLeuLysAlaGluGluLeuLeuLeuLysIle
      ----------------------------------------------------------
  241 CTAGGATTTGAAGTGAAATGCTTTAATGATCTTAAAGCAGAAGAACTACTGCTCAAAATT
      GATCCTAAACTTCACTTTACGAAATTACTAGAATTTCGTCTTCTTGATGACGAGTTTTAA
   +1 HisGluValSerThrValSerHisAlaAspAlaAspCysPheValCysValPheLeuSer
      ----------------------------------------------------------
  301 CATGAGGTGTCAACTGTTAGCCACGCAGATGCCGATTGCTTTGTGTGTGTCTTCCTGAGC
      GTACTCCACAGTTGACAATCGGTGCGTCTACGGCTAACGAAACACACACAGAAGGACTCG
   +1 HisGlyGluGlyAsnHisIleTyrAlaTyrAspAlaLysIleGluIleGlnThrLeuThr
      ----------------------------------------------------------
  361 CATGGCGAAGGCAATCACATTTATGCATATGATGCTAAAATCGAAATTCAGACATTAACT
      GTACCGCTTCCGTTAGTGTAAATACGTATACTACGATTTTAGCTTTAAGTCTGTAATTGA
   +1 GlyLeuPheLysGlyAspLysCysHisSerLeuValGlyLysProLysIlePheIleIle
      ----------------------------------------------------------
  421 GGCTTGTTCAAAGGAGACAAGTGTCACAGCCTGGTTGGAAAACCCAAGATATTTATCATC
      CCGAACAAGTTTCCTCTGTTCACAGTGTCGGACCAACCTTTTGGGTTCTATAAATAGTAG
   +1 GlnAlaCysArgGlyAsnGlnHisAspValProValIleProLeuAspValValAspAsn
      ----------------------------------------------------------
  481 CAGGCATGTCGGGGAAACCAGCACGATGTGCCAGTCATTCCTTTGGATGTAGTAGATAAT
      GTCCGTACAGCCCCTTTGGTCGTGCTACACGGTCAGTAAGGAAACCTACATCATCTATTA
   +1 GlnThrGluLysLeuAspThrAsnIleThrGluValAspAlaAlaSerValTyrThrLeu
      ----------------------------------------------------------
  541 CAGACAGAGAAGTTGGACACCAACATAACTGAGGTGGATGCAGCCTCCGTTTACACGCTG
      GTCTGTCTCTTCAACCTGTGGTTGTATTGACTCCACCTACGTCGGAGGCAAATGTGCGAC
   +1 ProAlaGlyAlaAspPheLeuMetCysTyrSerValAlaGluGlyTyrTyrSerHisArg
      ----------------------------------------------------------
  601 CCTGCTGGAGCTGACTTCCTCATGTGTTACTCTGTTGCAGAAGGATATTATTCTCACCGG
      GGACGACCTCGACTGAAGGAGTACACAATGAGACAACATCTTCCTATAATAAGACGTGCC
```

Fig. 16B

CASPASE 6

```
  +1 GluThrValAsnGlySerTrpTyrIleGlnAspLeuCysGluMetLeuGlyLysTyrGly
     ------------------------------------------------------------
661  GAAACTGTGAACGGCTCATGGTACATTCAAGATTTGTGTGAGATGTTGGGAAAATATGGC
     CTTTGACACTTGCCGAGTACCATGTAAGTTCTAAACACACTCTACAACCCTTTTATACCG
  +1 SerSerLeuGluPheThrGluLeuLeuThrLeuValAsnArgLysValSerGlnArgArg
     ------------------------------------------------------------
721  TCCTCCTTAGAGTTCACAGAACTCCTCACACTGGTGAACAGGAAAGTTTCTCAGCGCCGA
     AGGAGGAATCTCAAGTGTCTTGAGGAGTGTGACCACTTGTCCTTTCAAAGAGTCGCGGCT
  +1 ValAspPheCysLysAspProSerAlaIleGlyLysLysGlnValProCysPheAlaSer
     ------------------------------------------------------------
781  GTGGACTTTTGCAAAGACCCAAGTGCAATTGGAAAGAAGCAGGTTCCCTGTTTTGCCTCA
     CACCTGAAAACGTTTCTGGGTTCACGTTAACCTTTCTTCGTCCAAGGGACAAAACGGAGT
  +1 MetLeuThrLysLysLeuHisPhePheProLysSerAsn
     ---------------------------------------->
841  ATGCTAACTAAAAAGCTGCATTTCTTTCCAAAATCTAATTAAN
     TACGATTGATTTTTCGACGTAAAGAAAGGTTTTAGATTAATTN
```

Fig. 17A

CASPASE 7

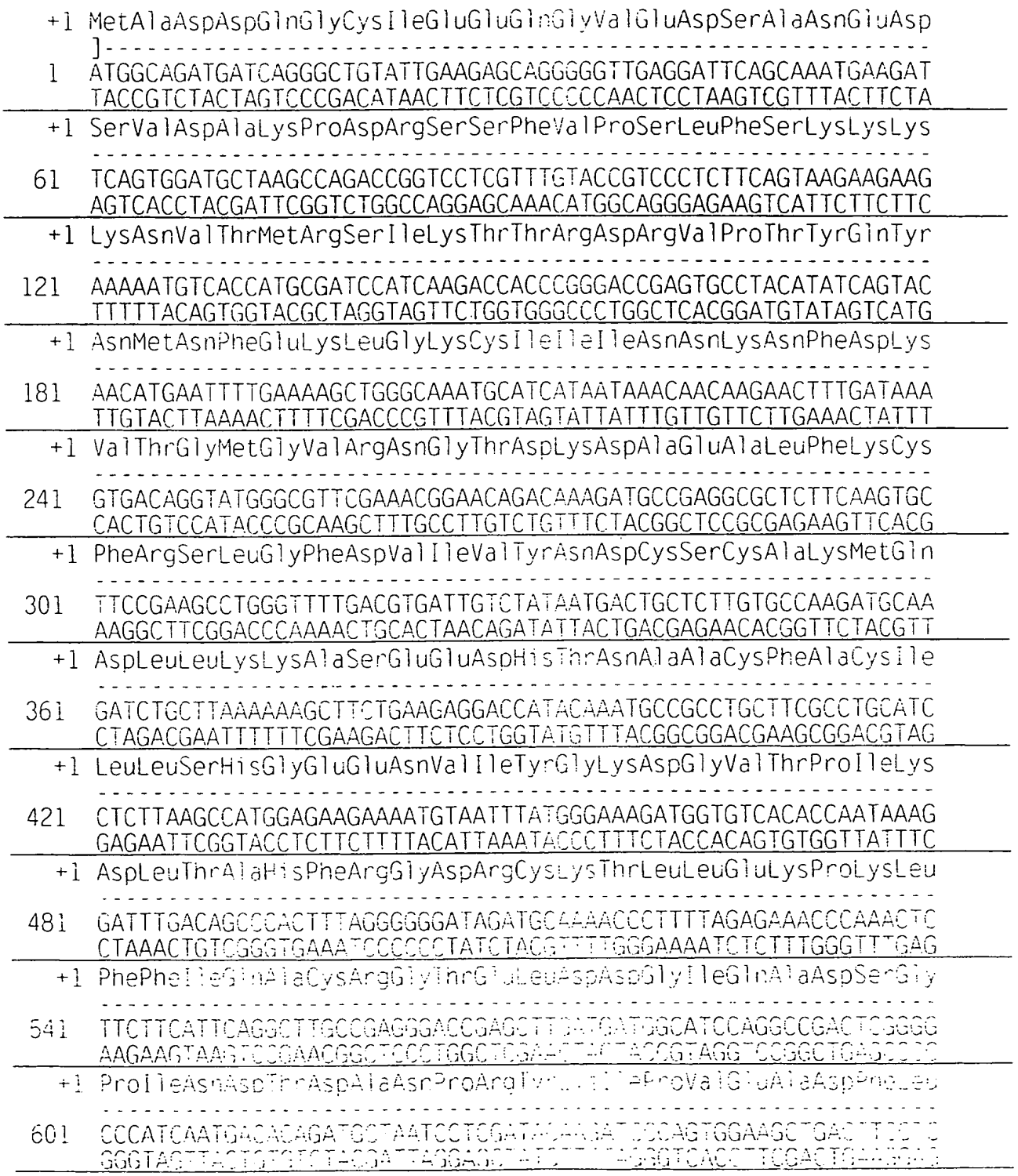

Fig. 17B

CASPASE 7

```
   +1 PheAlaTyrSerThrValProGlyTyrTyrSerTrpArgSerProGlyArgGlySerTrp
      ------------------------------------------------------------
661   TTCGCCTATTCCACGGTTCCAGGCTATTACTCGTGGAGGAGCCCAGGAAGAGGCTCCTGG
      AAGCGGATAAGGTGCCAAGGTCCGATAATGAGCACCTCCTCGGGTCCTTCTCCGAGGACC
   +1 PheValGlnAlaLeuCysSerIleLeuGluGluHisGlyLysAspLeuGluIleMetGln
      ------------------------------------------------------------
721   TTTGTGCAAGCCCTCTGCTCCATCCTGGAGGAGCACGGAAAAGACCTGGAAATCATGCAG
      AAACACGTTCGGGAGACGAGGTAGGACCTCCTCGTGCCTTTTCTGGACCTTTAGTACGTC
   +1 IleLeuThrArgValAsnAspArgValAlaArgHisPheGluSerGlnSerAspAspPro
      ------------------------------------------------------------
781   ATCCTCACCAGGGTGAATGACAGAGTTGCCAGGCACTTTGAGTCTCAGTCTGATGACCCA
      TAGGAGTGGTCCCACTTACTGTCTCAACGGTCCGTGAAACTCAGAGTCAGACTACTGGGT
   +1 HisPheHisGluLysLysGlnIleProCysValValSerMetLeuThrLysGluLeuTyr
      ------------------------------------------------------------
841   CACTTCCATGAGAAGAAGCAGATCCCCTGTGTGGTCTCCATGCTCACCAAGGAACTCTAC
      GTGAAGGTACTCTTCTTCGTCTAGGGGACACACCAGAGGTACGAGTGGTTCCTTGAGATG
   +1 PheSerGln
      -------->
901   TTCAGTCAATAGN
      AAGTCAGTTATCN
```

Fig. 18A

CASPASE 8

```
+1  MetAspPheSerArgAsnLeuTyrAspIleGlyGluGlnLeuAspSerGluAspLeuAla
    ]----------------------------------------------------------
1   ATGGACTTCAGCAGAAATCTTTATGATATTGGGGAACAACTGGACAGTGAAGATCTGGCC
    TACCTGAAGTCGTCTTTAGAAATACTATAACCCCTTGTTGACCTGTCACTTCTAGACCGG
+1  SerLeuLysPheLeuSerLeuAspTyrIleProGlnArgLysGlnGluProIleLysAsp
    ----------------------------------------------------------
61  TCCCTCAAGTTCCTGAGCCTGGACTACATTCCGCAAAGGAAGCAAGAACCCATCAAGGAT
    AGGGAGTTCAAGGACTCGGACCTGATGTAAGGCGTTTCCTTCGTTCTTGGGTAGTTCCTA
+1  AlaLeuMetLeuPheGlnArgLeuGlnGluLysArgMetLeuGluGluSerAsnLeuSer
    ----------------------------------------------------------
121 GCCTTGATGTTATTCCAGAGACTCCAGGAAAAGAGAATGTTGGAGGAAAGCAATCTGTCC
    CGGAACTACAATAAGGTCTCTGAGGTCCTTTTCTCTTACAACCTCCTTTCGTTAGACAGG
+1  PheLeuLysGluLeuLeuPheArgIleAsnArgLeuAspLeuLeuIleThrTyrLeuAsn
    ----------------------------------------------------------
181 TTCCTGAAGGAGCTGCTCTTCCGAATTAATAGACTGGATTTGCTGATTACCTACCTAAAC
    AAGGACTTCCTCGACGAGAAGGCTTAATTATCTGACCTAAACGACTAATGGATGGATTTG
+1  ThrArgLysGluGluMetGluArgGluLeuGlnThrProGlyArgAlaGlnIleSerAla
    ----------------------------------------------------------
241 ACTAGAAAGGAGGAGATGGAAAGGGAACTTCAGACACCAGGCAGGGCTCAAATTTCTGCC
    TGATCTTTCCTCCTCTACCTTTCCCTTGAAGTCTGTGGTCCGTCCCGAGTTTAAAGACGG
+1  TyrArgPheHisPheCysArgMetSerTrpAlaGluAlaAsnSerGlnCysGlnThrGln
    ----------------------------------------------------------
301 TACAGGTTCCACTTCTGCCGCATGAGCTGGGCTGAAGCAAACAGCCAGTGCCAGACACAG
    ATGTCCAAGGTGAAGACGGCGTACTCGACCCGACTTCGTTTGTCGGTCACGGTCTGTGTC
+1  SerValProPheTrpArgArgValAspHisLeuLeuIleArgValMetLeuTyrGlnIle
    ----------------------------------------------------------
361 TCTGTACCTTTCTGGCGGAGGGTCGATCATCTATTAATAAGGGTCATGCTCTATCAGATT
    AGACATGGAAAGACCGCCTCCCAGCTAGTAGATAATTATTCCCAGTACGAGATAGTCTAA
+1  SerGluGluValSerArgSerGluLeuArgSerPheLysPheLeuLeuGlnGluGluIle
    ----------------------------------------------------------
421 TCAGAAGAAGTGAGCAGATCAGAATTGAGGTCTTTTAAGTTTCTTTTGCAAGAGGAAATC
    AGTCTTCTTCACTCGTCTAGTCTTAACTCCAGAAAATTCAAAGAAAACGTTCTCCTTTAG
+1  SerLysCysLysLeuAspAspAspMetAsnLeuLeuAspIlePheIleGluMetGluLys
    ----------------------------------------------------------
481 TCCAAATGCAAACTGGATGATGACATGAACCTGCTGGATATTTTCATAGAGATGGAGAAG
    AGGTTTACGTTTGACCTACTACTGTACTTGGACGACCTATAAAAGTATCTCTACCTCTTC
+1  ArgValIleLeuGlyGluGlyLysLeuAspIleLeuLysArgValCysAlaGlnIleAsn
    ----------------------------------------------------------
541 AGGGTCATCCTGGGAGAAGGAAAGTTGGACATCCTGAAAAGAGTCTGTGCCCAAATCAAC
    TCCCAGTAGGACCCTCTTCCTTTCAACCTGTAGGACTTTTCTCAGACACGGGTTTAGTTG
+1  LysSerLeuLeuLysIleIleAsnAspTrpGluGluPheSerLysGlyGluGluLeuGlu
    ----------------------------------------------------------
601 AAGAGCCTGCTGAAGATAATCAACGACTATGAAGAATTCAGCAAAGGGGAGGAGTTGGAG
    TTCTCGGACGACTTCTATTAGTTGCTGATACTTCTTAAGTCGTTCCCCTCCTCAACCTC
```

Fig. 18B

CASPASE 8

```
   +1 GlyValMetThrIleSerAspSerProArgGluGlnAspSerGluSerGlnThrLeuAsp
      ------------------------------------------------------------
 661  GGGGTAATGACAATCTCGGACTCTCCAAGAGAACAGGATAGTGAATCACAGACTTTGGAC
      CCCCATTACTGTTAGAGCCTGAGAGGTTCTCTTGTCCTATCACTTAGTGTCTGAAACCTG
   +1 LysValTyrGlnMetLysSerLysProArgGlyTyrCysLeuIleIleAsnAsnHisAsn
      ------------------------------------------------------------
 721  AAAGTTTACCAAATGAAAAGCAAACCTCGGGGATACTGTCTGATCATCAACAATCACAAT
      TTTCAAATGGTTTACTTTTCGTTTGGAGCCCCTATGACAGACTAGTAGTTGTTAGTGTTA
   +1 PheAlaLysAlaArgGluLysValProLysLeuHisSerIleArgAspArgAsnGlyThr
      ------------------------------------------------------------
 781  TTTGCAAAAGCACGGGAGAAAGTGCCCAAACTTCACAGCATTAGGGACAGGAATGGAACA
      AAACGTTTTCGTGCCCTCTTTCACGGGTTTGAAGTGTCGTAATCCCTGTCCTTACCTTGT
   +1 HisLeuAspAlaGlyAlaLeuThrThrThrPheGluGluLeuHisPheGluIleLysPro
      ------------------------------------------------------------
 841  CACTTGGATGCAGGGGCTTTGACCACGACCTTTGAAGAGCTTCATTTTGAGATCAAGCCC
      GTGAACCTACGTCCCCGAAACTGGTGCTGGAAACTTCTCGAAGTAAAACTCTAGTTCGGG
   +1 HisHisAspCysThrValGluGlnIleTyrGluIleLeuLysIleTyrGlnLeuMetAsp
      ------------------------------------------------------------
 901  CACCATGACTGCACAGTAGAGCAAATCTATGAGATTTTGAAAATCTACCAACTCATGGAC
      GTGGTACTGACGTGTCATCTCGTTTAGATACTCTAAAACTTTTAGATGGTTGAGTACCTG
   +1 HisSerAsnMetAspCysPheIleCysCysIleLeuSerHisGlyAspLysGlyIleIle
      ------------------------------------------------------------
 961  CACAGTAACATGGACTGCTTCATCTGCTGTATCCTCTCCCATGGAGACAAGGGCATCATC
      GTGTCATTGTACCTGACGAAGTAGACGACATAGGAGAGGGTACCTCTGTTCCCGTAGTAG
   +1 TyrGlyThrAspGlyGlnGluAlaProIleTyrGluLeuThrSerGlnPheThrGlyLeu
      ------------------------------------------------------------
1021  TATGGCACTGATGGACAGGAGGCCCCCATCTATGAGCTGACATCTCAGTTCACTGGTTTG
      ATACCGTGACTACCTGTCCTCCGGGGGTAGATACTCGACTGTAGAGTCAAGTGACCAAAC
   +1 LysCysProSerLeuAlaGlyLysProLysValPhePheIleGlnAlaCysGlnGlyAsp
      ------------------------------------------------------------
1081  AAGTGCCCTTCCCTTGCTGGAAAACCCAAAGTGTTTTTTATTCAGGCTTGTCAGGGGGAT
      TTCACGGGAAGGGAACGACCTTTTGGGTTTCACAAAAAATAAGTCCGAACAGTCCCCCTA
   +1 AsnTyrGlnLysGlyIleProValGluThrAspSerGluGluGlnProTyrLeuGluMet
      ------------------------------------------------------------
1141  AACTACCAGAAAGGTATACCTGTTGAGACTGATTCAGAGGAGCAACCCTATTTAGAAATG
      TTGATGGTCTTTCCATATGGACAACTCTGACTAAGTCTCCTCGTTGGGATAAATCTTTAC
   +1 AspLeuSerSerProGlnThrArgTyrIleProAspGluAlaAspPheLeuLeuGlyMet
      ------------------------------------------------------------
1201  GATTTATCATCACCTCAAACGAGATATATCCCGGATGAGGCTGACTTTCTGCTGGGGATG
      CTAAATAGTAGTGGAGTTTGCTCTATATAGGGCCTACTCCGACTGAAAGACGACCCCTAC
   +1 AlaThrValAsnAsnCysValSerTyrArgAsnProAlaGluGlyThrTrpTyrIleGln
      ------------------------------------------------------------
1261  GCCACTGTGAATAACTGTGTTTCCTACCGAAACCCTGCAGAGGGAACCTGGTACATCCAG
      CGGTGACACTTATTGACACAAAGGATGGCTTTGGGACGTCTCCCTTGGACCATGTAGGTC
```

Fig. 18C

CASPASE 8

```
   +1 SerLeuCysGlnSerLeuArgGluArgCysProArgGlyAspAspIleLeuThrIleLeu
      ------------------------------------------------------------
1321  TCACTTTGCCAGAGCCTGAGAGAGCGATGTCCTCGAGGCGATGATATTCTCACCATCCTG
      AGTGAAACGGTCTCGGACTCTCTCGCTACAGGAGCTCCGCTACTATAAGAGTGGTAGGAC
   +1 ThrGluValAsnTyrGluValSerAsnLysAspAspLysLysAsnMetGlyLysGlnMet
      ------------------------------------------------------------
1381  ACTGAAGTGAACTATGAAGTAAGCAACAAGGATGACAAGAAAAACATGGGGAAACAGATG
      TGACTTCACTTGATACTTCATTCGTTGTTCCTACTGTTCTTTTTGTACCCCTTTGTCTAC
   +1 ProGlnProThrPheThrLeuArgLysLysLeuValPheProSerAsp
      ------------------------------------------------>
1441  CCTCAGCCTACTTTCACACTAAGAAAAAAACTTGTCTTCCCTTCTGATTGANN
      GGAGTCGGATGAAAGTGTGATTCTTTTTTTGAACAGAAGGGAAGACTAACTNN
```

Fig. 19A

CASPASE 9

```
  +1 MetAspGluAlaAspArgArgLeuLeuArgArgCysArgLeuArgLeuValGluGluLeu
     ]----------------------------------------------------------
   1 ATGGACGAAGCGGATCGGCGGCTCCTGCGGCGGTGCCGGCTGCGGCTGGTGGAAGAGCTG
     TACCTGCTTCGCCTAGCCGCCGAGGACGCCGCCACGGCCGACGCCGACCACCTTCTCGAC
  +1 GlnValAspGlnLeuTrpAspAlaLeuLeuSerSerGluLeuPheArgProHisMetIle
     ----------------------------------------------------------
  61 CAGGTGGACCAGCTCTGGGACGCCCTGCTGAGCAGCGAGCTGTTCAGGCCCCATATGATC
     GTCCACCTGGTCGAGACCCTGCGGGACGACTCGTCGCTCGACAAGTCCGGGGTATACTAG
  +1 GluAspIleGlnArgAlaGlySerGlySerArgArgAspGlnAlaArgGlnLeuIleIle
     ----------------------------------------------------------
 121 GAGGACATCCAGCGGGCAGGCTCTGGATCTCGGCGGGATCAGGCCAGGCAGCTGATCATA
     CTCCTGTAGGTCGCCCGTCCGAGACCTAGAGCCGCCCTAGTCCGGTCCGTCGACTAGTAT
  +1 AspLeuGluThrArgGlySerGlnAlaLeuProLeuPheIleSerCysLeuGluAspThr
     ----------------------------------------------------------
 181 GATCTGGAGACTCGAGGGAGTCAGGCTCTTCCTTTGTTCATCTCCTGCTTAGAGGACACA
     CTAGACCTCTGAGCTCCCTCAGTCCGAGAAGGAAACAAGTAGAGGACGAATCTCCTGTGT
  +1 GlyGlnAspMetLeuAlaSerPheLeuArgThrAsnArgGlnAlaAlaLysLeuSerLys
     ----------------------------------------------------------
 241 GGCCAGGACATGCTGGCTTCGTTTCTGCGAACTAACAGGCAAGCAGCAAAGTTGTCGAAG
     CCGGTCCTGTACGACCGAAGCAAAGACGCTTGATTGTCCGTTCGTCGTTTCAACAGCTTC
  +1 ProThrLeuGluAsnLeuThrProValValLeuArgProGluIleArgLysProGluVal
     ----------------------------------------------------------
 301 CCAACCCTAGAAAACCTTACCCCAGTGGTGCTCAGACCAGAGATTCGCAAACCAGAGGTT
     GGTTGGGATCTTTTGGAATGGGGTCACCACGAGTCTGGTCTCTAAGCGTTTGGTCTCCAA
  +1 LeuArgProGluThrProArgProValAspIleGlySerGlyGlyPheGlyAspValGly
     ----------------------------------------------------------
 361 CTCAGACCGGAAACACCCAGACCAGTGGACATTGGTTCTGGAGGATTTGGTGATGTCGGT
     GAGTCTGGCCTTTGTGGGTCTGGTCACCTGTAACCAAGACCTCCTAAACCACTACAGCCA
  +1 AlaLeuGluSerLeuArgGlyAsnAlaAspLeuAlaTyrIleLeuSerMetGluProCys
     ----------------------------------------------------------
 421 GCTCTTGAGAGTTTGAGGGGAAATGCAGATTTGGCTTACATCCTGAGCATGGAGCCCTGT
     CGAGAACTCTCAAACTCCCCTTTACGTCTAAACCGAATGTAGGACTCGTACCTCGGGACA
  +1 GlyHisCysLeuIleIleAsnAsnValAsnPheCysArgGluSerGlyLeuArgThrArg
     ----------------------------------------------------------
 481 GGCCACTGCCTCATTATCAACAATGTGAACTTCTGCCGTGAGTCCGGGCTCCGCACCCGC
     CCGGTGACGGAGTAATAGTTGTTACACTTGAAGACGGCACTCAGGCCCGAGGCGTGGGCG
  +1 ThrGlySerAsnIleAspCysGluLysLeuArgArgArgPheSerSerProHisPheMet
     ----------------------------------------------------------
 541 ACTGGCTCCAACATCGACTGTGAGAAGTTGCGGCGTCGCTTCTCCTCGCCGCATTTCATG
     TGACCGAGGTTGTAGCTGACACTCTTCAACGCCGCAGCGAAGAGGAGCGGCGTAAAGTAC
  +1 ValGluValLysGlyAspLeuThrAlaLysLysMetValLeuAlaLeuLeuGluLeuAla
     ----------------------------------------------------------
 601 GTGGAGGTGAAGGGCGACCTGACTGCCAAGAAAATGGTGCTGGCTTTGCTGGAGCTGGCG
     CACCTCCACTTCCCGCTGGACTGACGGTTCTTTTACCACGACCGAAACGACCTCGACCGC
```

Fig. 19B

CASPASE 9

```
   +1 ArgGlnAspHisGlyAlaLeuAspCysCysValValValIleLeuSerHisGlyCysGln
      ------------------------------------------------------------
661   CGGCAGGACCACGGTGCTCTGGACTGCTGCGTGGTGGTCATTCTCTCTCACGGCTGTCAG
      GCCGTCCTGGTGCCACGAGACCTGACGACGCACCACCAGTAAGAGAGAGTGCCGACAGTC
   +1 AlaSerHisLeuGlnPheProGlyAlaValTyrGlyThrAspGlyCysProValSerVal
      ------------------------------------------------------------
721   GCCAGCCACCTGCAGTTCCCAGGGGCTGTCTACGGCACAGATGGATGCCCTGTGTCGGTC
      CGGTCGGTGGACGTCAAGGGTCCCCGACAGATGCCGTGTCTACCTACGGGACACAGCCAG
   +1 GluLysIleValAsnIlePheAsnGlyThrSerCysProSerLeuGlyGlyLysProLys
      ------------------------------------------------------------
781   GAGAAGATTGTGAACATCTTCAATGGGACCAGCTGCCCCAGCCTGGGAGGAAAGCCCAAG
      CTCTTCTAACACTTGTAGAAGTTACCCTGGTCGACGGGGTCGGACCCTCCTTTCGGGTTC
   +1 LeuPhePheIleGlnAlaCysGlyGlyGluGlnLysAspHisGlyPheGluValAlaSer
      ------------------------------------------------------------
841   CTCTTTTTCATCCAGGCCTGTGGTGGGGAGCAGAAAGACCATGGGTTTGAGGTGGCCTCC
      GAGAAAAAGTAGGTCCGGACACCACCCCTCGTCTTTCTGGTACCCAAACTCCACCGGAGG
   +1 ThrSerProGluAspGluSerProGlySerAsnProGluProAspAlaThrProPheGln
      ------------------------------------------------------------
901   ACTTCCCCTGAAGACGAGTCCCCTGGCAGTAACCCCGAGCCAGATGCCACCCCGTTCCAG
      TGAAGGGGACTTCTGCTCAGGGGACCGTCATTGGGGCTCGGTCTACGGTGGGGCAAGGTC
   +1 GluGlyLeuArgThrPheAspGlnLeuAspAlaIleSerSerLeuProThrProSerAsp
      ------------------------------------------------------------
961   GAAGGTTTGAGGACCTTCGACCAGCTGGACGCCATATCTAGTTTGCCCACACCCAGTGAC
      CTTCCAAACTCCTGGAAGCTGGTCGACCTGCGGTATAGATCAAACGGGTGTGGGTCACTG
   +1 IlePheValSerTyrSerThrPheProGlyPheValSerTrpArgAspProLysSerGly
      ------------------------------------------------------------
1021  ATCTTTGTGTCCTACTCTACTTTCCCAGGTTTTGTTTCCTGGAGGGACCCCAAGAGTGGC
      TAGAAACACAGGATGAGATGAAAGGGTCCAAAACAAAGGACCTCCCTGGGGTTCTCACCG
   +1 SerTrpTyrValGluThrLeuAspAspIlePheGluGlnTrpAlaHisSerGluAspLeu
      ------------------------------------------------------------
1081  TCCTGGTACGTTGAGACCCTGGACGACATCTTTGAGCAGTGGGCTCACTCTGAAGACCTG
      AGGACCATGCAACTCTGGGACCTGCTGTAGAAACTCGTCACCCGAGTGAGACTTCTGGAC
   +1 GlnSerLeuLeuLeuArgValAlaAsnAlaValSerValLysGlyIleTyrLysGlnMet
      ------------------------------------------------------------
1141  CAGTCCCTCCTGCTTAGGGTCGCTAATGCTGTTTCGGTGAAAGGGATTTATAAACAGATG
      GTCAGGGAGGACGAATCCCAGCGATTACGACAAAGCCACTTTCCCTAAATATTTGTCTAC
   +1 ProGlyCysPheAsnPheLeuArgLysLysLeuPhePheLysThrSer
      ------------------------------------------------>
1201  CCTGGTTGCTTTAATTTCCTCCGGAAAAAACTTTTCTTTAAAAGATCATAAN
      GGACCAACGAAATTAAAGGAGGCCTTTTTTGAAAAGAAATTTTGTAGTATTN
```

Fig. 20A

CASPASE 10

```
   +1 MetLysSerGlnGlyGlnHisTrpTyrSerSerSerAspLysAsnCysLysValSerPhe
      ]-------------------------------------------------------------
    1 ATGAAATCTCAAGGTCAACATTGGTATTCCAGTTCAGATAAAAACTGTAAAGTGAGCTTT
      TACTTTAGAGTTCCAGTTGTAACCATAAGGTCAAGTCTATTTTTGACATTTCACTCGAAA
   +1 ArgGluLysLeuLeuIleIleAspSerAsnLeuGlyValGlnAspValGluAsnLeuLys
      -------------------------------------------------------------
   61 CGTGAGAAGCTTCTGATTATTGATTCAAACCTGGGGGTCCAAGATGTGGAGAACCTCAAG
      GCACTCTTCGAAGACTAATAACTAAGTTTGGACCCCCAGGTTCTACACCTCTTGGAGTTC
   +1 PheLeuCysIleGlyLeuValProAsnLysLysLeuGluLysSerSerSerAlaSerAsp
      -------------------------------------------------------------
  121 TTTCTCTGCATAGGATTGGTCCCCAACAAGAAGCTGGAGAAGTCCAGCTCAGCCTCAGAT
      AAAGAGACGTATCCTAACCAGGGGTTGTTCTTCGACCTCTTCAGGTCGAGTCGGAGTCTA
   +1 ValPheGluHisLeuLeuAlaGluAspLeuLeuSerGluGluAspProPhePheLeuAla
      -------------------------------------------------------------
  181 GTTTTTGAACATCTCTTGGCAGAGGATCTGCTGAGTGAGGAAGACCCTTTCTTCCTGGCA
      CAAAAACTTGTAGAGAACCGTCTCCTAGACGACTCACTCCTTCTGGGAAAGAAGGACCGT
   +1 GluLeuLeuTyrIleIleArgGlnLysLysLeuLeuGlnHisLeuAsnCysThrLysGlu
      -------------------------------------------------------------
  241 GAACTCCTCTATATCATACGGCAGAAGAAGCTGCTGCAGCACCTCAACTGTACCAAAGAG
      CTTGAGGAGATATAGTATGCCGTCTTCTTCGACGACGTCGTGGAGTTGACATGGTTTCTC
   +1 GluValGluArgLeuLeuProThrArgGlnArgValSerLeuPheArgAsnLeuLeuTyr
      -------------------------------------------------------------
  301 GAAGTGGAGCGACTGCTGCCCACCCGACAAAGGGTTTCTCTGTTTAGAAACCTGCTCTAC
      CTTCACCTCGCTGACGACGGGTGGGCTGTTTCCCAAAGAGACAAATCTTTGGACGAGATG
   +1 GluLeuSerGluGlyIleAspSerGluAsnLeuLysAspMetIlePheLeuLeuLysAsp
      -------------------------------------------------------------
  361 GAACTGTCAGAAGGCATTGACTCAGAGAACTTAAAGGACATGATCTTCCTTCTGAAAGAC
      CTTGACAGTCTTCCGTAACTGAGTCTCTTGAATTTCCTGTACTAGAAGGAAGACTTTCTG
   +1 SerLeuProLysThrGluMetThrSerLeuSerPheLeuAlaPheLeuGluLysGlnGly
      -------------------------------------------------------------
  421 TCGCTTCCCAAAACTGAAATGACCTCCCTAAGTTTCCTGGCATTTCTAGAGAAACAAGGT
      AGCGAAGGGTTTTGACTTTACTGGAGGGATTCAAAGGACCGTAAAGATCTCTTTGTTCCA
   +1 LysIleAspGluAspAsnLeuThrCysLeuGluAspLeuCysLysThrValValProLys
      -------------------------------------------------------------
  481 AAAATAGATGAAGATAATCTGACATGCCTGGAGGACCTCTGCAAAACAGTTGTACCTAAA
      TTTTATCTACTTCTATTAGACTGTACGGACCTCCTGGAGACGTTTTGTCAACATGGATTT
   +1 LeuLeuArgAsnIleGluLysTyrLysArgGluLysAlaIleGlnIleValThrProPro
      -------------------------------------------------------------
  541 CTTTTGAGAAACATAGAGAAATACAAAAGAGAGAAAGCTATCCAGATAGTGACACCTCCT
      GAAAACTCTTTGTATCTCTTTATGTTTTCTCTCTTTCGATAGGTCTATCACTGTGGAGGA
   +1 ValAspLysGluAlaGluSerTyrGlnGlyGluGluLeuValSerGlnThrAspVal
      -------------------------------------------------------------
  601 GTAGACAAGGAAGCCGAGTCGTATCAAGGAGAGGAAGAACTAGTTTCCCAAACAGATGTT
      CATCTGTTCCTTCGGCTCAGCATAGTTCCTCTCCTTCTTGATCAAAGGGTTTGTCTACAA
```

Fig. 20B

CASPASE 10

```
   +1 LysThrPheLeuGluAlaLeuProArgAlaAlaValTyrArgMetAsnArgAsnHisArg
      ------------------------------------------------------------
661   AAGACATTCTTGGAAGCCTTACCGAGGGCAGCTGTGTACAGGATGAATCGGAACCACAGA
      TTCTGTAAGAACCTTCGGAATGGCTCCCGTCGACACATGTCCTACTTAGCCTTGGTGTCT
   +1 GlyLeuCysValIleValAsnAsnHisSerPheThrSerLeuLysAspArgGlnGlyThr
      ------------------------------------------------------------
721   GGCCTCTGTGTCATTGTCAACAACCACAGCTTTACCTCCCTGAAGGACAGACAAGGAACC
      CCGGAGACACAGTAACAGTTGTTGGTGTCGAAATGGAGGGACTTCCTGTCTGTTCCTTGG
   +1 HisLysAspAlaGluIleLeuSerHisValPheGlnTrpLeuGlyPheThrValHisIle
      ------------------------------------------------------------
781   CATAAAGATGCTGAGATCCTGAGTCATGTGTTCCAGTGGCTTGGGTTCACAGTGCATATA
      GTATTTCTACGACTCTAGGACTCAGTACACAAGGTCACCGAACCCAAGTGTCACGTATAT
   +1 HisAsnAsnValThrLysValGluMetGluMetValLeuGlnLysGlnLysCysAsnPro
      ------------------------------------------------------------
841   CACAATAATGTGACGAAAGTGGAAATGGAGATGGTCCTGCAGAAGCAGAAGTGCAATCCA
      GTGTTATTACACTGCTTTCACCTTTACCTCTACCAGGACGTCTTCGTCTTCACGTTAGGT
   +1 AlaHisAlaAspGlyAspCysPheValPheCysIleLeuThrHisGlyArgPheGlyAla
      ------------------------------------------------------------
901   GCCCATGCCGACGGGGACTGCTTCGTGTTCTGTATTCTGACCCATGGGAGATTTGGAGCT
      CGGGTACGGCTGCCCCTGACGAAGCACAAGACATAAGACTGGGTACCCTCTAAACCTCGA
   +1 ValTyrSerSerAspGluAlaLeuIleProIleArgGluIleMetSerHisPheThrAla
      ------------------------------------------------------------
961   GTCTACTCTTCGGATGAGGCCCTCATTCCCATTCGGGAGATCATGTCTCACTTCACAGCC
      CAGATGAGAAGCCTACTCCGGGAGTAAGGGTAAGCCCTCTAGTACAGAGTGAAGTGTCGG
   +1 LeuGlnCysProArgLeuAlaGluLysProLysLeuPhePheIleGlnAlaCysGlnGly
      ------------------------------------------------------------
1021  CTGCAGTGCCCTAGACTGGCTGAAAAACCTAAACTCTTTTTCATCCAGGCCTGCCAAGGT
      GACGTCACGGGATCTGACCGACTTTTTGGATTTGAGAAAAAGTAGGTCCGGACGGTTCCA
   +1 GluGluIleGlnProSerValSerIleGluAlaAspAlaLeuAsnProGluGlnAlaPro
      ------------------------------------------------------------
1081  GAAGAGATACAGCCTTCCGTATCCATCGAAGCAGATGCTCTGAACCCTGAGCAGGCACCC
      CTTCTCTATGTCGGAAGGCATAGGTAGCTTCGTCTACGAGACTTGGGACTCGTCCGTGGG
   +1 ThrSerLeuGlnAspSerIleProAlaGluAlaAspPheLeuLeuGlyLeuAlaThrVal
      ------------------------------------------------------------
1141  ACTTCCCTGCAGGACAGTATTCCTGCCGAGGCTGACTTCCTACTTGGTCTGGCCACTGTC
      TGAAGGGACGTCCTGTCATAAGGACGGCTCCGACTGAAGGATGAACCAGACCGGTGACAG
   +1 ProGlyTyrValSerPheArgHisValGluGluGlySerTrpTyrIleGlnSerLeuCys
      ------------------------------------------------------------
1201  CCAGGCTATGTATCCTTTCGGCATGTGGAGGAAGGCAGCTGGTATATTCAGTCTCTGTGT
      GGTCCGATACATAGGAAAGCCGTACACCTCCTTCCGTCGACCATATAAGTCAGAGACACA
   +1 AsnHisLeuLysLysLeuValProArgHisGluAspIleLeuSerIleLeuThrAlaVal
      ------------------------------------------------------------
1261  AATCATCTGAAGAAATTGGTCCCAAGACATGAAGACATCTTATCCATCCTCACTGCTGTC
      TTAGTAGACTTCTTTAACCAGGGTTCTGTACTTCTGTAGAATAGGTAGGAGTGACGACAG
```

Fig. 20C

CASPASE 10

```
   +1 AsnAspAspValSerArgArgValAspLysGlnGlyThrLysLysGlnMetProGlnPro
      ------------------------------------------------------------
1321  AACGATGATGTGAGTCGAAGAGTGGACAAACAGGGAACAAAGAAACAGATGCCCCAGCCT
      TTGCTACTACACTCAGCTTCTCACCTGTTTGTCCCTTGTTTCTTTGTCTACGGGGTCGGA
   +1 AlaPheThrLeuArgLysLysLeuValPheProValProLeuAspAlaLeuSerIle
      ----------------------------------------------------------->
1381  GCTTTCACACTAAGGAAAAAACTAGTATTCCCTGTGCCCCTGGATGCACTTTCAATATAG
      CGAAAGTGTGATTCCTTTTTTGATCATAAGGGACACGGGGACCTACGTGAAAGTTATATC
```

Fig. 21A

Rev-caspase-3

```
Met Ile Glu Thr Asp Ser Gly Val Asp Asp Asp Met Ala Cys His Lys Ile Pro
Val Glu Ala Asp Phe Leu Tyr Ala Tyr Ser Thr Ala Pro Gly Tyr Tyr Ser Trp
Arg Asn Ser Lys Asp Gly Ser Trp Phe Ile Gln Ser Leu Cys Ala Met Leu Lys
Gln Tyr Ala Asp Lys Leu Glu Phe Met His Ile Leu Thr Arg Val Asn Arg Lys
Val Ala Thr Glu Phe Glu Ser Phe Ser Phe Asp Ala Thr Phe His Ala Lys Lys
Gln Ile Pro Cys Ile Val Ser Met Leu Thr Lys Glu Leu Tyr Phe Tyr His Asp
Glu Val Asp Gly Gly Ser Pro Met Glu Asn Thr Glu Asn Ser Val Asp Ser Lys
Ser Ile Lys Asn Leu Glu Pro Lys Ile Ile His Gly Ser Glu Ser Met Asp Ser
Gly Ile Ser Leu Asp Asn Ser Tyr Lys Met Asp Tyr Pro Glu Met Gly Leu Cys
Ile Ile Ile Asn Asn Lys Asn Phe His Lys Ser Thr Gly Met Thr Ser Arg Ser
Gly Thr Asp Val Asp Ala Ala Asn Leu Arg Glu Thr Phe Arg Asn Leu Lys Tyr
Glu Val Arg Asn Lys Asn Asp Leu Thr Arg Glu Glu Ile Val Glu Leu Met Arg
Asp Val Ser Lys Glu Asp His Ser Lys Arg Ser Ser Phe Val Cys Val Leu Leu
Ser His Gly Glu Glu Gly Ile Ile Phe Gly Thr Asn Gly Pro Val Asp Leu Lys
Lys Ile Thr Asn Phe Phe Arg Gly Asp Arg Cys Arg Ser Leu Thr Gly Lys Pro
Lys Leu Phe Ile Ile Gln Ala Cys Arg Gly Thr Glu Leu Asp Cys Gly Ile Glu
Thr Asp
```

Fig. 21B

Uncleavable Rev-caspase-3

Met Ile Glu Thr Asp Ser Gly Val Asp Asp Asp Met Ala Cys His Lys Ile Pro
Val Glu Ala Asp Phe Leu Tyr Ala Tyr Ser Thr Ala Pro Gly Tyr Tyr Ser Trp
Arg Asn Ser Lys Asp Gly Ser Trp Phe Ile Gln Ser Leu Cys Ala Met Leu Lys
Gln Tyr Ala Asp Lys Leu Glu Phe Met His Ile Leu Thr Arg Val Asn Arg Lys
Val Ala Thr Glu Phe Glu Ser Phe Ser Phe Asp Ala Thr Phe His Ala Lys Lys
Gln Ile Pro Cys Ile Val Ser Met Leu Thr Lys Glu Leu Tyr Phe Tyr His Gly
Ser Pro Met Glu Asn Thr Glu Asn Ser Val Ala Ser Lys Ser Ile Lys Asn Leu
Glu Pro Lys Ile Ile His Gly Ser Glu Ser Met Ala Ser Gly Ile Ser Leu Asp
Asn Ser Tyr Lys Met Asp Tyr Pro Glu Met Gly Leu Cys Ile Ile Ile Asn Asn
Lys Asn Phe His Lys Ser Thr Gly Met Thr Ser Arg Ser Gly Thr Asp Val Asp
Ala Ala Asn Leu Arg Glu Thr Phe Arg Asn Leu Lys Tyr Glu Val Arg Asn Lys
Asn Asp Leu Thr Arg Glu Glu Ile Val Glu Leu Met Arg Asp Val Ser Lys Glu
Asp His Ser Lys Arg Ser Ser Phe Val Cys Val Leu Leu Ser His Gly Glu Glu
Gly Ile Ile Phe Gly Thr Asn Gly Pro Val Asp Leu Lys Lys Ile Thr Asn Phe
Phe Arg Gly Asp Arg Cys Arg Ser Leu Thr Gly Lys Pro Lys Leu Phe Ile Ile
Gln Ala Cys Arg Gly Thr Glu Leu Asp Cys Gly Ile Glu Thr Asp

Fig. 21C

> Rev-caspase-6

Met Val Glu Ile Asp Ala Ala Ser Val Tyr Thr Leu Pro Ala Gly Ala Asp Phe
Leu Met Cys Tyr Ser Val Ala Glu Gly Tyr Tyr Ser His Arg Glu Thr Val Asn
Gly Ser Trp Tyr Ile Gln Asp Leu Cys Glu Met Leu Gly Lys Tyr Gly Ser Ser
Leu Glu Phe Thr Glu Leu Leu Thr Leu Val Asn Arg Lys Val Ser Gln Arg Arg
Val Asp Phe Cys Lys Asp Pro Ser Ala Ile Gly Lys Lys Gln Val Pro Cys Phe
Ala Ser Met Leu Thr Lys Lys Leu His Phe Phe Pro Lys Ser Asn Leu Glu His
His His His His His Val Glu Ile Asp Gly Gly Ser Pro Met Ser Ser Ala Ser
Gly Leu Arg Arg Gly His Pro Ala Gly Gly Glu Glu Asn Met Thr Glu Thr Asp
Ala Phe Tyr Lys Arg Glu Met Phe Asp Pro Ala Glu Lys Tyr Lys Met Asp His
Arg Arg Arg Gly Ile Ala Leu Ile Phe Asn His Glu Arg Phe Phe Trp His Leu
Thr Leu Pro Glu Arg Arg Gly Thr Cys Ala Asp Arg Asp Asn Leu Thr Arg Arg
Phe Ser Asp Leu Gly Phe Glu Val Lys Cys Phe Asn Asp Leu Lys Ala Glu Glu
Leu Leu Leu Lys Ile His Glu Val Ser Thr Val Ser His Ala Asp Ala Asp Cys
Phe Val Cys Val Phe Leu Ser His Gly Glu Gly Asn His Ile Tyr Ala Tyr Asp
Ala Lys Ile Glu Ile Gln Thr Leu Thr Gly Leu Phe Lys Gly Asp Lys Cys His
Ser Leu Val Gly Lys Pro Lys Ile Phe Ile Ile Gln Ala Cys Arg Gly Asn Gln
His Asp Val Pro Val Ile Pro Leu Asp Val Val Asp

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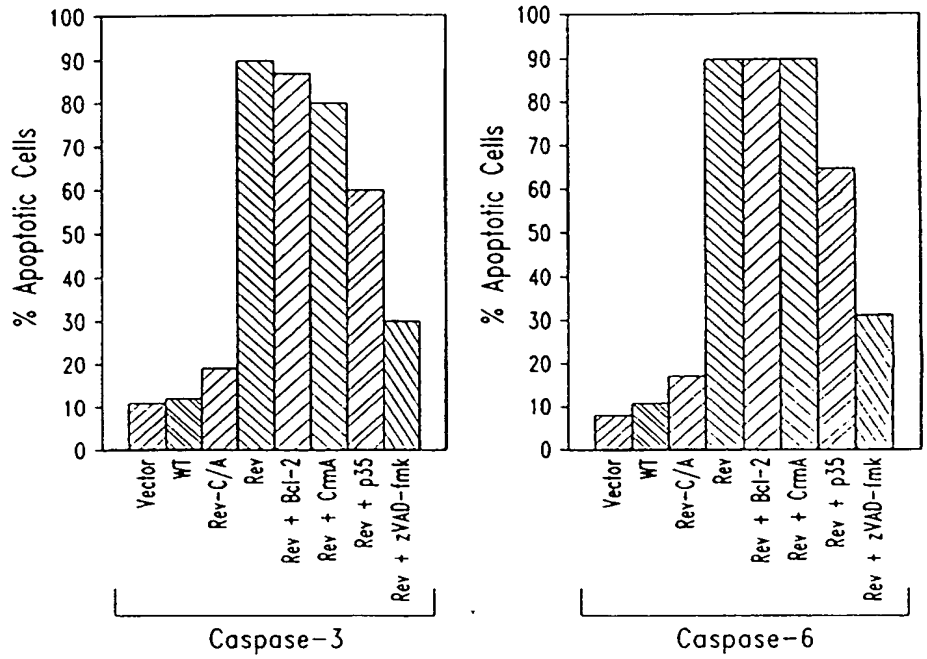
(54) Title: RECOMBINANT, ACTIVE CASPASES AND USES THEREOF



(57) Abstract

Rev-caspases comprising a primary product in which the small subunit is N terminal to the large subunit are provided. Rev-caspases are used for screening and identifying caspase inhibitors and enhancers. Rev caspase genes can be delivered to cells for gene therapy.

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US 99/00632

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 35 and 36 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat. al Application No
PCT/US 99/00632

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9531535 A	23-11-1995	US 5492824 A	20-02-1996
		CA 2189581 A	23-11-1995
		EP 0754224 A	22-01-1997
		JP 10503084 T	24-03-1998
EP 533350 A	24-03-1993	CA 2076159 A	17-02-1993
		DE 69229252 D	01-07-1999
		JP 11069972 A	16-03-1999
		JP 5227961 A	07-09-1993

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/00632

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/57 C12N9/64 C12N1/21 C12N5/10 C12Q1/37
A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 31535 A (BASF AG) 23 November 1995 (1995-11-23) page 2, paragraph 3 - page 5, paragraph 2; examples	1-36
A	EP 0 533 350 A (MRCK & CO., INC.) 24 March 1993 (1993-03-24) page 5, line 3 - page 13, line 2; examples 23-31	1-36

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[X] Further documents are listed in the continuation of box C

[X] Patent family members are listed in annex

Special categories of cited documents

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Date of the actual completion of the international search	Date of mailing of the international search report
3 August 1999	16/08/1999

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/00632

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No
P,X	SRINIVASA M. SRINIVASULA ET AL.: "Generation of constitutively active recombinant Caspases-3 and -6 by rearrangement of their subunits" JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 273, no. 17, 24 April 1998 (1998-04-24), pages 10107-10111, XP002111125 MD US abstract page 10108, left-hand column, paragraph 2 - page 10110, right-hand column, paragraph 3	1-36